

## 5 BINDING POLYPEPTIDES FOR B LYMPHOCYTE STIMULATOR PROTEIN (BLyS)

## FIELD OF THE INVENTION

10 The present invention relates to polypeptides that bind to B lymphocyte stimulator protein (BLyS). Such polypeptides have uses for example, in the detection, isolation, and/or purification of BLyS. The invention also relates to nucleic acid molecules encoding these BLyS binding polypeptides, vectors and host cells containing these nucleic acids, and methods for producing the same.

## BACKGROUND OF THE INVENTION

20 B lymphocyte stimulator (BLyS) is a member of the tumor necrosis factor ("TNF") superfamily that induces both *in vivo* and *in vitro* B cell proliferation and differentiation (Moore et al., Science, 285: 260-263 (1999)). BLyS is distinguishable from other B cell growth and differentiation factors such as IL-2, IL-4, IL-5, IL-6, IL-7, IL-13, IL-15, CD40L, or CD27L (CD70) by its monocyte-specific gene and protein expression pattern and its specific receptor distribution and biological activity on B lymphocytes. BLyS expression is not detected on natural killer ("NK") cells, T cells or B cells, but is restricted to cells of myeloid origin. BLyS expression on resting monocytes is upregulated by interferon-gamma (IFN-gamma). The gene encoding BLyS has been mapped to chromosome 13q34.

25 BLyS is expressed as a 285 amino acid type II membrane-bound polypeptide and a soluble 152 amino acid polypeptide (Moore et al., 1999, *supra*). The membrane-bound form of BLyS has a predicted transmembrane spanning domain between amino acid residues 47 and 73. The NH<sub>2</sub>-terminus of the soluble form of BLyS begins at Ala<sup>134</sup> of the membrane-bound form of BLyS. Both the soluble and membrane-bound forms of the protein form homotrimers. Soluble  
30 recombinant BLyS has been shown to induce *in vitro* proliferation of murine splenic B cells and to bind to a cell-surface receptor on these cells (Moore et al., 1999, *supra*). Soluble BLyS administration to mice has been shown to result in an increase in the proportion of CD45R<sup>dull</sup>,

Ly6D<sup>bright</sup> (also known as ThB) B cells and an increase in serum IgM and IgA levels (Moore et al., 1999, *supra*). Thus, BLyS displays a B cell tropism in both its receptor distribution and biological activity.

Based on its expression pattern and biological activity, BLyS has been suggested to be involved in the exchange of signals between B cells and monocytes or their differentiated progeny. The restricted expression patterns of BLyS receptor and ligand suggest that BLyS may function as a regulator of T cell-independent responses in a manner analogous to that of CD40 and CD40L in T cell-dependent antigen activation.

Accordingly, molecules that specifically bind BLyS would find a variety of uses in the study of the BLyS cytokine, in the manufacture and purification of BLyS in commercial and medically pure quantities, and in the development new therapeutic or diagnostic reagents.

## SUMMARY OF THE INVENTION

The present invention provides new polypeptides and families of polypeptides that specifically bind to B lymphocyte stimulator protein (BLyS) and/or BLyS-like polypeptides. In particular, the invention encompasses polypeptides that specifically bind to a polypeptide or polypeptide fragment of human BLyS (SEQ ID NOS:173 and/or 174) or BLyS expressed on human monocytes; murine BLyS (SEQ ID NOS:175 and/or 176) or BLyS expressed on murine monocytes; rat BLyS (either the soluble forms as given in SEQ ID NOS:177, 178, 179 and/or 180 or in a membrane associated form, e.g., on the surface of rat monocytes); or monkey BLyS (e.g., the monkey BLyS polypeptides of SEQ ID NOS:181 and/or 182, the soluble form of monkey BLyS, or BLyS expressed on monkey monocytes), preferably human BLyS.

In specific preferred embodiments, the BLyS binding polypeptides of the invention bind BLyS and/or BLyS-like polypeptides with high affinity. In other embodiments, the BLyS binding polypeptides of the invention reversibly bind BLyS and/or BLyS-like polypeptides. In still other embodiments, the BLyS binding polypeptides of the invention irreversibly bind BLyS and/or BLyS-like polypeptides.

The cysteine residues in certain polypeptides according to the invention are believed to form a disulfide bond, which would cause the polypeptide containing these cysteine residues to form a stable loop structure under non-reducing conditions. Especially preferred BLyS binding

polypeptides of the invention are polypeptide molecules that comprise amino acid sequences that form stable loop structures or other stable structures that bind BLYS or BLYS-like polypeptides.

In specific embodiments, the invention relates to BLYS binding polypeptides comprising, or alternatively consisting of, an amino acid sequence selected from the group consisting of SEQ ID NOs:1-12, 20-172, and 186-444, preferably SEQ ID NOs:163-172 or 436-444 as referred to above and in Tables 1-8, 14 and 15 and in Examples 2, 5 and 6 below. Analysis of the sequences of the BLYS binding polypeptides isolated as described herein shows a strong selection for polypeptides containing the tetrapeptide Asp-Xaa-Leu-Thr (SEQ ID NO:446), and therefore in its broadest aspects, the present invention relates to polypeptides capable of binding to BLYS comprising the polypeptide Asp-Xaa-Leu-Thr (SEQ ID NO:446), where Xaa is Pro, Ser, Thr, Phe, Leu, Tyr, Cys, or Ala (preferably Pro or Ser).

Seven consensus sequences (SEQ ID NOs:1-7) have been determined based on the specific BLYS binding polypeptides shown in Tables 1-8. In specific embodiments, BLYS binding polypeptides of the invention comprise one or more of these sequences. Such preferred BLYS binding polypeptides include polypeptides with the potential to form a cyclic or loop structure between invariant Cys residues comprising, or alternatively consisting of, an amino acid sequence selected from A-E (SEQ ID NOs:1-5):

(A)  $X_1-X_2-X_3-Cys-X_5-Phe-X_7-Trp-Glu-Cys-X_{11}-X_{12}-X_{13}$  (SEQ ID NO:1),

wherein

$X_1$  is Ala, Asn, Lys, or Ser;

$X_2$  is Ala, Glu, Met, Ser, or Val;

$X_3$  is Ala, Asn, Lys, or Pro (preferably Lys);

$X_5$  is Phe, Trp, or Tyr (preferably Tyr);

$X_7$  is Pro or Tyr (preferably Pro);

$X_{11}$  is Ala, Gln, His, Phe, or Val;

$X_{12}$  is Asn, Gln, Gly, His, Ser, or Val; and

$X_{13}$  is Ala, Asn, Gly, Ile, Pro, or Ser,

wherein said polypeptide binds BLYS and/or BLYS-like polypeptides; or

(B)  $X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-Cys-X_{12}-X_{13}-X_{14}$  (SEQ ID NO:2),

wherein

X<sub>1</sub> is Ala, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, or is absent;

X<sub>2</sub> is Ala, Asn, Asp, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X<sub>3</sub> is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Asp);

X<sub>5</sub> is Asp, Ile, Leu, or Tyr (preferably Asp or Leu);

X<sub>6</sub> is Arg, Asp, Glu, His, Ile, Leu, Lys, Phe, Pro, Tyr, or Val (preferably Glu or Leu);

X<sub>7</sub> is His, Leu, Lys, or Phe (preferably His or Leu);

X<sub>8</sub> is Leu, Pro, or Thr (preferably Thr or Pro);

10 X<sub>9</sub> is Arg, Asn, Gly, His, Ile, Lys, Met, or Trp (preferably Lys);

X<sub>10</sub> is Ala, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Ser, Trp, Tyr, or Val;

X<sub>12</sub> is Asp, Gln, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Trp, Tyr, or Val;

X<sub>13</sub> is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val; and

X<sub>14</sub> is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Trp, Tyr, Val, or is absent, wherein said polypeptide binds BLYS and/or BLYS-like polypeptides; or

(C) X<sub>1</sub>-X<sub>2</sub>-X<sub>3</sub>-Cys-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-X<sub>10</sub>-X<sub>11</sub>-Cys-X<sub>13</sub>-X<sub>14</sub>-X<sub>15</sub> (SEQ ID NO:3),

wherein

X<sub>1</sub> is Ala, Arg, Asn, Asp, Leu, Lys, Phe, Pro, Ser, or Thr;

X<sub>2</sub> is Asn, Asp, Gln, His, Ile, Lys, Pro, Thr, or Trp;

X<sub>3</sub> is Ala, Arg, Asn, Gln, Glu, His, Phe, Pro, or Thr (preferably Ala);

X<sub>5</sub> is Asn, Asp, Pro, Ser, or Thr (preferably Asp);

X<sub>6</sub> is Arg, Asp, Ile, Leu, Met, Pro, or Val (preferably Ile);

X<sub>7</sub> is Ala, Ile, Leu, Pro, Thr, or Val (preferably Val or Leu);

25 X<sub>8</sub> is Asn, His, Ile, Leu, Lys, Phe, or Thr (preferably Thr);

X<sub>9</sub> is Asn, Glu, Gly, His, Leu, Lys, Met, Pro, or Thr (preferably Leu);

X<sub>10</sub> is Arg, Asn, Asp, Gln, Glu, Gly, Ile, Lys, Met, Pro, Ser, or Trp;

X<sub>11</sub> is Arg, Glu, Gly, Lys, Phe, Ser, Trp, or Tyr (preferably Ser);

X<sub>13</sub> is Gln, Glu, Ile, Leu, Phe, Pro, Ser, Tyr, or Val (preferably Val);

30 X<sub>14</sub> is Asn, Gly, Ile, Phe, Pro, Thr, Trp, or Tyr; and

X<sub>15</sub> is Asn, Asp, Glu, Leu, Lys, Met, Pro, or Thr (preferably Glu or Pro),



wherein said polypeptide binds BLYS and/or BLYS-like polypeptides; or

(D)  $X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-Cys-X_{14}-X_{15}-X_{16}$  (SEQ ID

NO:4),

wherein

$X_1$  is Asn, Asp, His, Leu, Phe, Pro, Ser, Tyr, or is absent (preferably Ser);

$X_2$  is Arg, Asn, Asp, His, Phe, Ser, or Trp (preferably Arg);

$X_3$  is Asn, Asp, Leu, Pro, Ser, or Val (preferably Asn or Asp);

$X_5$  is Asp, Gln, His, Ile, Leu, Lys, Met, Phe, or Thr;

$X_6$  is His, Ile, Leu, Met, Phe, Pro, Trp, or Tyr;

$X_7$  is Asp, His, Leu, or Ser (preferably Asp);

$X_8$  is Ala, Arg, Asp, Glu, Leu, Phe, Pro, or Thr (preferably Glu or Pro);

$X_9$  is Ala, Arg, Asn, or Leu (preferably Leu);

$X_{10}$  is Ile, Leu, Met, Pro, Ser, or Thr (preferably Thr);

$X_{11}$  is Ala, Arg, Asn, Gly, His, Lys, Ser, or Tyr;

$X_{12}$  is Ala, Arg, Asn, Gln, Leu, Met, Ser, Trp, Tyr, or Val;

$X_{14}$  is Asp, Gly, Leu, Phe, Tyr, or Val (preferably Leu);

$X_{15}$  is Asn, His, Leu, Pro, or Tyr (preferably His, Leu or Pro); and

$X_{16}$  is Asn, Asp, His, Phe, Ser, or Tyr, (preferably Asp or Ser),

wherein said polypeptide binds BLYS and/or BLYS-like polypeptides; or

(E)  $X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14}-Cys-X_{16}-X_{17}-X_{18}$

(SEQ ID NO:5),

wherein

$X_1$  is Arg, Asp, Gly, His, Leu, Phe, Pro, Ser, Trp, Tyr, or is absent (preferably Arg);

$X_2$  is Ala, Arg, Asn, Asp, Gly, Pro, Ser, or is absent (preferably Asn, Asp, Gly, or Pro);

$X_3$  is Arg, Asn, Gln, Glu, Gly, Lys, Met, Pro, Trp or Val (preferably Gly or Met);

$X_5$  is Arg, Asn, Gln, Glu, His, Leu, Phe, Pro, Trp, Tyr, or Val (preferably Trp, Tyr, or Val);

$X_6$  is Arg, Asp, Gln, Gly, Ile, Lys, Phe, Thr, Trp or Tyr (preferably Asp);

$X_7$  is Ala, Arg, Asp, Glu, Gly, Leu, Ser, or Tyr (preferably Asp);

$X_8$  is Asp, Gln, Glu, Leu, Met, Phe, Pro, Ser, or Tyr (preferably Leu);

$X_9$  is Asp, Leu, Pro, Thr, or Val (preferably Leu or Thr);

$X_{10}$  is Arg, Gln, His, Ile, Leu, Lys, Met, Phe, Thr, Trp or Tyr (preferably Lys or Thr);

X<sub>11</sub> is Ala, Arg, Asn, Gln, Glu, His, Leu, Lys, Met, or Thr (preferably Arg or Leu);

X<sub>12</sub> is Ala, Asn, Gln, Gly, Leu, Lys, Phe, Pro, Thr, Trp, or Tyr (preferably Thr or Trp);

X<sub>13</sub> is Ala, Arg, Gln, His, Lys, Met, Phe, Pro, Thr, Trp, or Tyr (preferably Met or Phe);

X<sub>14</sub> is Arg, Gln, Glu, Gly, His, Leu, Met, Phe, Pro, Ser, Thr, Tyr, or Val (preferably Val);

5 X<sub>16</sub> is Arg, Asp, Gly, His, Lys, Met, Phe, Pro, Ser, or Trp (preferably Met);

X<sub>17</sub> is Arg, Asn, Asp, Gly, His, Phe, Pro, Ser, Trp or Tyr, (preferably Arg, His, or Tyr); and

X<sub>18</sub> is Ala, Arg, Asn, Asp, His, Leu, Phe, or Trp (preferably His or Asn),

wherein said polypeptide binds BLyS and/or BLyS-like polypeptides.

10 Additional preferred embodiments include linear polypeptides comprising, or alternatively consisting of, an amino acid sequence selected from F and G (SEQ ID NOs:6 and 7):

(F) X<sub>1</sub>-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-X<sub>10</sub>-X<sub>11</sub>-X<sub>12</sub> (SEQ ID NO:6),

wherein

X<sub>1</sub> is Ala, Arg, Gly, His, Leu, Lys, Met, Phe, Trp, Tyr, or Val (preferably Gly, Tyr, or Val);

X<sub>2</sub> is Ala, Arg, Gln, His, Ile, Leu, Phe, Thr, Trp, or Tyr (preferably His or Tyr);

X<sub>3</sub> is Ala, Asp, Lys, Phe, Thr, Trp or Tyr (preferably Asp or Tyr);

X<sub>4</sub> is Arg, Asp, Gln, Lys, Met, Phe, Pro, Ser, Tyr, or Val (preferably Asp or Gln);

X<sub>5</sub> is Asp, Leu, Lys, Phe, Pro, Ser, or Val (preferably Leu or Ser);

X<sub>6</sub> is His, Ile, Leu, Pro, Ser, or Thr (preferably Leu or Thr);

20 X<sub>7</sub> is Arg, Gly, His, Leu, Lys, Met, or Thr (preferably Lys or Thr);

X<sub>8</sub> is Ala, Arg, Asn, Ile, Leu, Lys, Met, or Thr (preferably Leu or Lys);

X<sub>9</sub> is Ala, Asn, Arg, Asp, Glu, Gly, His, Leu, Met, Ser, Trp, Tyr, or Val (preferably Met or Ser);

X<sub>10</sub> is Ile, Leu, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Thr or Leu);

X<sub>11</sub> is Ala, Arg, Gly, His, Ile, Leu, Lys, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Pro or Thr);

25 and

X<sub>12</sub> is Arg, Asp, His, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Arg or Pro),

wherein said polypeptide binds BLyS and/or BLyS-like polypeptides; or

(G) X<sub>1</sub>-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-X<sub>10</sub>-X<sub>11</sub>-X<sub>12</sub>-X<sub>13</sub> (SEQ ID NO:7),

wherein

30 X<sub>1</sub> is Asp, Gln, Glu, Gly, His, Lys, Met, or Trp (preferably Glu, Lys);

X<sub>2</sub> is Arg, Gln, His, Ile, Leu, or Pro (preferably His or Pro);

X<sub>3</sub> is Asp, Gly, Ile, Lys, Thr, Tyr or Val (preferably Tyr);

X<sub>4</sub> is Asn, Asp, Gln, Glu, Met, Pro, Ser, or Tyr (preferably Asp or Gln);

X<sub>5</sub> is Asn, Asp, His, Ile, Leu, Met, Pro, Thr or Val (preferably Asn or Thr);

X<sub>6</sub> is Asp, Glu, His, Leu, Lys, Pro, or Val (preferably Asp or Pro);

5 X<sub>7</sub> is Arg, Asn, Gln, His, Ile, Leu, Met, Pro, or Thr (preferably Ile or Pro);

X<sub>8</sub> is Gln, Gly, His, Leu, Met, Ser, or Thr (preferably Leu or Thr);

X<sub>9</sub> is Asn, Gln, Gly, His, Leu, Lys, Ser, or Thr (preferably Lys);

X<sub>10</sub> is Ala, Gly, Ile, Leu, Lys, Met, or Phe (preferably Gly or Met);

X<sub>11</sub> is Ala, Glu, His, Ile, Leu, Met, Ser, Thr, Trp, Tyr, or Val (preferably Ala or Thr);

10 X<sub>12</sub> is Arg, Gln, Glu, Gly, His, Ile, Lys, Tyr, or Val (preferably Arg or His); and

X<sub>13</sub> is Arg, Asn, Glu, His, Ile, Ser, Thr, Trp, or Val (preferably His),

wherein said polypeptide binds BLYS and/or BLYS-like polypeptides.

Said polypeptides may have additional amino acids attached at either or both of the N- and C-terminal ends.

Examination of the sequence information and binding data from the isolates of libraries containing polypeptides with the potential to form loop structures (i.e., libraries designated TN6, TN7, TN8, TN9, TN10 and TN12) identifies a series of BLYS binding polypeptides that may form loop structures. In specific embodiments, BLYS binding polypeptides of the invention comprise, or alternatively consist of, an amino acid sequence selected from H-L (SEQ ID NOs:8-12):

(H) Cys-X<sub>2</sub>-Phe-X<sub>4</sub>-Trp-Glu-Cys (SEQ ID NO:8),

wherein

X<sub>2</sub> is Phe, Trp, or Tyr (preferably Tyr); and

X<sub>4</sub> is Pro or Tyr (preferably Pro); or

25 (I) Cys-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-Cys (SEQ ID NO:9),

wherein

X<sub>2</sub> is Asp, Ile, Leu, or Tyr (preferably Asp or Leu);

X<sub>3</sub> is Arg, Asp, Glu, His, Ile, Leu, Lys, Phe, Pro, Tyr, or Val (preferably Glu or Leu);

X<sub>4</sub> is His, Leu, Lys, or Phe (preferably His or Leu);

30 X<sub>5</sub> is Leu, Pro, or Thr (preferably Thr or Pro);

X<sub>6</sub> is Arg, Asn, Gly, His, Ile, Lys, Met, or Trp (preferably Lys); and

X<sub>7</sub> is Ala, Asn, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Ser, Trp, Tyr, or Val; or

(J) Cys-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-Cys (SEQ ID NO:10),

wherein

X<sub>2</sub> is Asn, Asp, Pro, Ser, or Thr (preferably Asp);

5 X<sub>3</sub> is Arg, Asp, Ile, Leu, Met, Pro, or Val (preferably Ile);

X<sub>4</sub> is Ala, Ile, Leu, Pro, Thr, or Val (preferably Val or Leu);

X<sub>5</sub> is Asn, His, Ile, Leu, Lys, Phe, or Thr (preferably Thr);

X<sub>6</sub> is Asn, Glu, Gly, His, Leu, Lys, Met, Pro, or Thr (preferably Leu);

X<sub>7</sub> is Arg, Asn, Asp, Gln, Glu, Gly, Ile, Lys, Met, Pro, Ser, or Trp;

10 X<sub>8</sub> is Arg, Glu, Gly, Lys, Phe, Ser, Trp, or Tyr (preferably Ser); or

(K) Cys-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-Cys (SEQ ID NO:11),

wherein

X<sub>2</sub> is Asp, Gln, His, Ile, Leu, Lys, Met, Phe, or Thr;

X<sub>3</sub> is His, Ile, Leu, Met, Phe, Pro, Trp, or Tyr;

X<sub>4</sub> is Asp, His, Leu, or Ser (preferably Asp);

X<sub>5</sub> is Ala, Arg, Asp, Glu, Leu, Phe, Pro, or Thr (preferably Glu or Pro);

X<sub>6</sub> is Ala, Arg, Asn, or Leu (preferably Leu);

X<sub>7</sub> is Ile, Leu, Met, Pro, Ser, or Thr (preferably Thr);

X<sub>8</sub> is Ala, Arg, Asn, Gly, His, Lys, Ser, or Tyr;

20 X<sub>9</sub> is Ala, Arg, Asn, Gln, Leu, Met, Ser, Trp, Tyr, or Val; or

(L) Cys-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-X<sub>10</sub>-X<sub>11</sub>-Cys (SEQ ID NO: 12),

wherein

X<sub>2</sub> is Arg, Asn, Gln, Glu, His, Leu, Phe, Pro, Trp, Tyr, or Val (preferably Trp, Tyr, or Val);

X<sub>3</sub> is Arg, Asp, Gln, Gly, Ile, Lys, Phe, Thr, Trp or Tyr (preferably Asp);

25 X<sub>4</sub> is Ala, Arg, Asp, Glu, Gly, Leu, Ser, or Tyr (preferably Asp);

X<sub>5</sub> is Asp, Gln, Glu, Leu, Met, Phe, Pro, Ser, or Tyr (preferably Leu);

X<sub>6</sub> is Asp, Leu, Pro, Thr, or Val (preferably Leu or Thr);

X<sub>7</sub> is Arg, Gln, His, Ile, Leu, Lys, Met, Phe, Thr, Trp or Tyr (preferably Lys or Thr);

X<sub>8</sub> is Ala, Arg, Asn, Gln, Glu, His, Leu, Lys, Met, or Thr (preferably Arg or Leu);

30 X<sub>9</sub> is Ala, Asn, Gln, Gly, Leu, Lys, Phe, Pro, Thr, Trp, or Tyr (preferably Thr or Trp);

X<sub>10</sub> is Ala, Arg, Gln, His, Lys, Met, Phe, Pro, Thr, Trp, or Tyr (preferably Met or Phe);

X<sub>11</sub> is Arg, Gln, Glu, Gly, His, Leu, Met, Phe, Pro, Ser, Thr, Tyr, or Val (preferably Val);  
wherein said polypeptides bind BLYS and/or BLYS-like polypeptides.

In additional preferred embodiments of the present invention, BLYS binding polypeptides comprise the following amino acid sequence M (SEQ ID NO:447):

(M) Ala-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-Asp-X<sub>6</sub>-Leu-Thr-X<sub>9</sub>-Leu-X<sub>11</sub>-X<sub>12</sub>-X<sub>13</sub>-X<sub>14</sub> (SEQ ID NO:447),

wherein

X<sub>2</sub> is Asn, Ser, Tyr, Asp, Phe, Ile, Gln, His, Pro, Lys, Leu, Met, Thr, Val, Glu, Ala, Gly, Cys, or Trp (i.e., any amino acid except Arg; preferably Asn);

X<sub>3</sub> is Trp, Glu, Lys, Cys, Leu, Ala, Arg, Gly, or Ser (preferably Trp);

X<sub>4</sub> is Tyr, Phe, Glu, Cys, Asn (preferably Tyr);

X<sub>6</sub> is Pro, Ser, Thr, Phe, Leu, Tyr, Cys, or Ala (preferably Pro or Ser);

X<sub>9</sub> is Lys, Asn, Gln, Gly, or Arg (preferably Lys);

X<sub>11</sub> is Trp, Ser, Thr, Arg, Cys, Tyr, or Lys (preferably Trp);

X<sub>12</sub> is Leu, Phe, Val, Ile, or His (preferably Leu);

X<sub>13</sub> is Pro, Leu, His, Ser, Arg, Asn, Gln, Thr, Val, Ala, Cys, Ile, Phe, or Tyr (i.e., not Asp, Glu, Gly, Lys, Met, or Trp; preferably Pro); and

X<sub>14</sub> is Asp, Glu, Asn, Val, His, Gln, Arg, Gly, Ser, Tyr, Ala, Cys, Lys, Ile, Thr or Leu (i.e., not Phe, Met, Pro, or Trp; preferably Asp, Val or Glu).

Preferred embodiments are polypeptides comprising a core sequence of the formula N:

(N) X<sub>1</sub>-X<sub>2</sub>-Asp-X<sub>4</sub>-Leu-Thr-X<sub>7</sub>-Leu-X<sub>9</sub>-X<sub>10</sub> (SEQ ID NO:448),

wherein

X<sub>1</sub> is Trp, Glu, Lys, Cys, Leu, Ala, Arg, Gly, or Ser (preferably Trp);

X<sub>2</sub> is Tyr, Phe, Glu, Cys, Asn (preferably Tyr);

X<sub>4</sub> is Pro, Ser, Thr, Phe, Leu, Tyr, Cys, or Ala (preferably Pro or Ser);

X<sub>7</sub> is Lys, Asn, Gln, Gly, or Arg (preferably Lys);

X<sub>9</sub> is Trp, Ser, Thr, Arg, Cys, Tyr, or Lys (preferably Trp); and

X<sub>10</sub> is Leu, Phe, Val, Ile, or His (preferably Leu).

Especially preferred BLYS binding polypeptides according to the present invention comprise the core peptide Trp-Tyr-Asp-Pro-Leu-Thr-Lys-Leu-Trp-Leu (SEQ ID NO:436).

The BLyS binding polypeptides described above may have additional amino acids attached at either or both of the N- and C-terminal ends.

A further embodiment of the present invention relates to a BLyS affinity maturation library, comprising a population of at least  $10^3$  polypeptides, preferably at least  $10^6$  polypeptides, more preferably at least  $10^9$  or more polypeptides, wherein the polypeptides of said population comprise the amino acid sequence:

Ala-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-Asp-X<sub>6</sub>-Leu-Thr-X<sub>9</sub>-Leu-X<sub>11</sub>-X<sub>12</sub>-X<sub>13</sub>-X<sub>14</sub> (SEQ ID NO:449),

wherein

X<sub>2</sub> is any amino acid;

X<sub>3</sub> is any amino acid;

X<sub>4</sub> is any amino acid;

X<sub>6</sub> is any amino acid;

X<sub>9</sub> is any amino acid;

X<sub>11</sub> is any amino acid;

X<sub>12</sub> is any amino acid;

X<sub>13</sub> is any amino acid; and

X<sub>14</sub> is any amino acid.

A preferred BLyS affinity maturation library will be produced such that the variable amino acid positions (i.e., positions 2, 3, 4, 6, 9, 11, 12, 13 and 14 in SEQ ID NO:449) will not be randomly variegated but will disproportionately be a single selected amino acid. Such a library may be produced by expression of a multiplicity of polynucleotides fitting the DNA template:

GCT NNN NNN NNN GAT NNN CTT ACT NNN CTC NNN NNN NNN NNN (SEQ ID NO:185), where each variable base (N) is A or C or G or T but one base in each instant is approximately 11-fold more probable at a given base position. One such DNA template, discussed in Example 6 below, is prepared so that the nucleotides of the DNA sequences are in the following proportions:

in DNA template (SEQ ID NO:185)		Proportion of Bases at Position			
Codon	Base Position	A	C	G	T
2	4	79%	7%	7%	7%
	5	79%	7%	7%	7%
	6	7%	7%	7%	79%
3	7	7%	7%	7%	79%
	8	7%	7%	79%	7%
	9	7%	7%	79%	7%
4	10	7%	7%	7%	79%
	11	79%	7%	7%	7%
	12	7%	7%	7%	79%
6	16	7%	7%	7%	79%
	17	7%	79%	7%	7%
	18	7%	7%	7%	79%
9	25	79%	7%	7%	7%
	26	79%	7%	7%	7%
	27	7%	7%	79%	7%
11	31	7%	7%	7%	79%
	32	7%	7%	79%	7%
	33	7%	7%	79%	7%
12	34	7%	79%	7%	7%
	35	7%	7%	7%	79%
	36	7%	7%	7%	79%
13	37	7%	79%	7%	7%
	38	7%	79%	7%	7%
	39	7%	7%	7%	79%
14	40	7%	7%	79%	7%
	41	79%	7%	7%	7%
	42	7%	7%	7%	79%

BLyS binding polypeptide molecules of the invention may also have an amino terminal (N-terminal) capping or functional group, such as an acetyl group, which, for example, blocks the amino terminal amino group from undesirable reactions or is useful in linking the BLyS binding polypeptide to another molecule, matrix, resin, or solid support. BLyS binding polypeptides of the invention may also have a carboxy terminal (C-terminal) capping or functional group, such as an amide group, which, for example, blocks the C-terminal carboxyl group from undesirable reactions or provides a functional group useful in conjugating the binding polypeptide to other molecules, matrices, resins, or solid supports. Preferably, the N- and/or C-terminal capping groups are polypeptide linker molecules. An especially preferred C-terminal linker molecule that is useful for immobilizing a BLyS binding polypeptide of the invention to a solid support or chromatographic matrix material comprises the amino acid sequence Pro-Gly-Pro-Glu-Gly-Gly-Gly-Lys (SEQ ID NO:13). Another useful C-terminal linker, e.g., for fluoresceinating peptides, is Gly-Gly-Lys (see Table 15).

The invention also encompasses BLyS binding polypeptides that have been modified, for example, to increase or decrease the stability of the molecule, while retaining the ability to bind BLyS and/or BLyS-like polypeptides. An example of a modified BLyS binding polypeptide of the invention is a polypeptide in which one of two cysteine residues is substituted with a non-naturally occurring amino acid that is capable of condensing with the remaining cysteine side chain to form a stable thioether bridge, thereby generating a cyclic BLyS binding polypeptide. Such cyclic thioether molecules of synthetic peptides may be routinely generated using techniques known in the art, e.g., as described in PCT publication WO 97/46251, incorporated herein by reference.

In another embodiment, the invention provides BLyS binding polypeptides of the invention attached, coupled, linked or adhered to a matrix or resin or solid support. Techniques for attaching, linking or adhering polypeptides to matrices, resins and solid supports are well known in the art. Suitable matrices, resins or solid supports for these materials may be any composition known in the art to which a BLyS binding polypeptide of the invention could be attached, coupled, linked, or adhered, including but not limited to, a chromatographic resin or matrix, such as SEPHAROSE-4 FF agarose beads, the wall or floor of a well in a plastic microtiter dish, such as used in an enzyme-linked immunosorbent assay (ELISA), or a silica based biochip. Materials useful as solid supports on which to immobilize binding polypeptides of the



invention include, but are not limited to, polyacrylamide, agarose, silica, nitrocellulose, paper, plastic, nylon, metal, and combinations thereof. A BLyS binding polypeptide of the invention may be immobilized on a matrix, resin or solid support material by a non-covalent association or by covalent bonding, using techniques known in the art. Preferably, a BLyS binding polypeptide of the invention is immobilized on a chromatography material such as SEPHAROSE-4 FF agarose. In an even more preferred embodiment, a BLyS binding polypeptide of the invention is coupled to a chromatography material using a linker molecule. A preferred linker molecule according to the present invention is a polypeptide comprising the amino acid sequence Pro-Gly-Pro-Glu-Gly-Gly-Gly-Lys (SEQ ID NO:13). Most preferably, the affinity chromatography material of the invention comprises a BLyS binding polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:163-172, which is linked to a chromatography material by a polypeptide linker molecule having the amino acid sequence Pro-Gly-Pro-Glu-Gly-Gly-Gly-Lys (SEQ ID NO:13). BLyS binding polypeptides of the invention attached, coupled, linked or adhered to a matrix or resin or other solid support are useful for methods of detecting, isolating and purifying BLyS and/or BLyS like polypeptides, particularly for purification of BLyS and/or BLyS like polypeptides by affinity chromatography.

In certain preferred embodiments, the BLyS binding polypeptides of the present invention or phage displaying such binding polypeptides, irreversibly bind the BLyS protein in its native, soluble trimeric form.

In certain preferred embodiments, the BLyS binding polypeptides of the present invention or phage displaying such binding polypeptides, reversibly bind the BLyS protein in its native, soluble trimeric form.

In a further embodiment, the present invention encompasses a composition of matter comprising isolated nucleic acids, preferably DNA, encoding a BLyS binding polypeptide of the invention. In a specific embodiment, nucleic acid molecules of the invention encode a BLyS binding polypeptide of the invention as provided in SEQ ID NOs:1-12, 20-172, and 186-444. In additional embodiments, nucleic acid molecules of the invention encode a polypeptide variant or fragment of a polypeptide comprising an amino acid sequence of SEQ ID NOs:1-12, 20-172, and 186-444. In a further additional embodiment, nucleic acid molecules of the invention encode a BLyS binding polypeptide, the complementary strand of which nucleic acid hybridizes to a polynucleotide sequence encoding a polypeptide described in Tables 1-8 and 14 and in Examples

2, 5 and 6 (SEQ ID NOs:1-12, 20-172, and 186-444), under stringent conditions, e.g., hybridization to filter-bound DNA in 6x sodium chloride/sodium citrate (SSC) at about 45° C followed by one or more washes in 0.2xSSC/0.1% SDS at about 50-65° C, under highly stringent conditions, e.g., hybridization to filter-bound nucleic acid in 6xSSC at about 45° C followed by one or more washes in 0.1xSSC/0.2% SDS at about 68° C, or under other stringent hybridization conditions which are known to those of skill in the art (see, for example, Ausubel, F.M. et al., eds. , 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York at pages 6.3.1-6.3.6 and 2.10.3).

The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules encoding the BLYS binding polypeptides of the present invention (as well as fragments and variants thereof), and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells. The invention further provides for the use of such recombinant vectors in the production of BLYS binding polypeptides by recombinant techniques.

The BLYS binding polypeptides, nucleic acids, transformed host cells, and genetically engineered viruses and phage of the invention (e.g., recombinant phage), have uses that include, but are not limited to, the detection, isolation, and purification of BLYS.

In another embodiment of the invention, recombinant bacteriophage displaying BLYS binding polypeptides on their surfaces are also provided. Such phage may be routinely generated using techniques known in the art and are useful, for example, as screening reagents and reagents for detecting BLYS.

In another embodiment, a BLYS binding polypeptide of the invention is used to detect or isolate BLYS or BLYS-like polypeptides in a solution. Such solutions include, but are not limited to, BLYS or BLYS-like polypeptides suspended or dissolved in water or a buffer solution as well as any fluid and/or cell obtained from an individual, biological fluid, body tissue, body cell, cell line, tissue culture, or other source which may contain BLYS or BLYS-like polypeptides, such as, cell culture medium, cell extracts, and tissue homogenates. Biological fluids include, but are not limited to, sera, plasma, lymph, blood, blood fractions, urine, synovial fluid, spinal fluid, saliva, and mucous.

In another embodiment, the present invention provides a method for detecting BLYS protein and/or BLYS-like polypeptide in a solution comprising, contacting the solution with a

BLyS binding polypeptide of the invention and detecting binding of BLyS or BLyS-like polypeptide to the BLyS binding polypeptide. The BLyS binding polypeptide may be either free or immobilized. Preferably, the BLyS binding polypeptide is a polypeptide immobilized on a solid surface or chromatographic material or the well of a plastic microtiter assay dish.

5 Another embodiment of the present invention is a method for isolating BLyS protein and/or BLyS-like polypeptide from a solution containing it, comprising:

- (a) contacting the solution with a BLyS binding polypeptide under conditions that permit binding of the BLyS and/or BLyS-like polypeptides to BLyS binding polypeptides, and
- 10 (b) separating BLyS binding polypeptides (and BLyS and/or BLyS-like polypeptides bound thereto) from the rest of the solution.

A further embodiment of the present invention is a method for isolating BLyS protein and/or BLyS-like polypeptide from a solution containing it, comprising:

- (a) contacting the solution with a BLyS binding polypeptide under conditions that permit binding of the BLyS and/or BLyS-like polypeptides to BLyS binding polypeptides,
- (b) separating the complex(es) formed by the BLyS binding polypeptide and BLyS and/or BLyS-like polypeptides from other components of the solution,
- (c) dissociating the BLyS binding polypeptide from the BLyS and/or BLyS-like polypeptides, and
- 20 (d) recovering the dissociated, BLyS and/or BLyS-like polypeptides.

In another embodiment, the invention provides kits containing a binding polypeptide of the invention for use in methods of detecting or isolating BLyS and/or BLyS-like polypeptides.

## 25 DEFINITIONS

In order that the invention may be clearly understood, the following terms are defined:

The term "recombinant" is used to describe non-naturally altered or manipulated nucleic acids, host cells transfected with exogenous nucleic acids, or polypeptide molecules that are expressed non-naturally, through manipulation of isolated nucleic acid (typically, DNA) and  
30 transformation or transfection of host cells. "Recombinant" is a term that specifically encompasses nucleic acid molecules that have been constructed *in vitro* using genetic engineering techniques, and

use of the term "recombinant" as an adjective to describe a molecule, construct, vector, cell, polypeptide or polynucleotide specifically excludes naturally occurring such molecules, constructs, vectors, cells, polypeptides or polynucleotides.

The term "bacteriophage" is defined as a bacterial virus containing a nucleic acid core and a protective shell built up by the aggregation of a number of different protein molecules. The terms "bacteriophage" and "phage" are synonymous and are used herein interchangeably.

The term "affinity ligand" is sometimes used herein and is synonymous with BLYS binding polypeptides of the invention.

The term "BLYS protein" as used herein encompasses both the membrane (e.g., SEQ ID NO:173) and soluble forms (e.g., SEQ ID NO:174). BLYS protein may be monomeric, dimeric, or trimeric or multivalent. Preferably, BLYS proteins are homotrimeric.

The term "BLYS-like polypeptide" as used herein encompasses natural BLYS or full-length recombinant BLYS as well as fragments and variants thereof, such as, a modified or truncated form of natural BLYS or full-length recombinant BLYS, which BLYS and BLYS-like polypeptide retain a BLYS functional activity. BLYS or BLYS fragments that may be specifically bound by the compositions of the invention include, but are not limited to, human BLYS (SEQ ID NOs:173 and/or 174) or BLYS expressed on human monocytes; murine BLYS (SEQ ID NOs:175 and/or 176) or BLYS expressed on murine monocytes; rat BLYS (either the soluble forms as given in SEQ ID NOs:177, 178, 179 and/or 180 or in a membrane associated form, e.g., on the surface of rat monocytes); or monkey BLYS (e.g., the monkey BLYS polypeptides of SEQ ID NOS:181 and/or 182, the soluble form of monkey BLYS, or BLYS expressed on monkey monocytes) or fragments thereof. Preferably compositions of the invention bind human BLYS (SEQ ID NOs:173 and/or 174) or fragments thereof. BLYS and BLYS-like polypeptides retain at least one functional activity of the natural or full-length BLYS, including but not limited to the following activities: binding to BLYS receptor (e.g., TACI (GenBank accession number AAC51790), and BCMA (GenBank accession number NP\_001183)), stimulating B cell proliferation, stimulating immunoglobulin secretion by B cells, stimulating the BLYS receptor signaling cascade and/or being bound by an anti-BLYS antibody or other BLYS binding polypeptide. Assays that can be used to determine the functional activities of BLYS or BLYS like polypeptides can readily be determined by one skilled in the art (e.g., see assays disclosed in Moore et al., 1999, *supra*) "BLYS-like polypeptides" also include fusion polypeptides in which all or a portion of BLYS is fused or

conjugated to another polypeptide. BLYS-like polypeptides that are fusion polypeptides retain at least one functional activity of BLYS, preferably the ability to stimulate B lymphocytes (see, for example, Moore et al., Science, 285: 260-263 (1999)), to bind the BLYS receptors (e.g., TACI or BCMA), and/or to be bound by an anti-BLYS antibody or other BLYS binding polypeptide. BLYS fusion polypeptides may be made by recombinant DNA techniques in which a gene or other polynucleotide coding sequence for BLYS or a fragment thereof is ligated in-frame (recombined) with the coding sequence of another protein or polypeptide. The resulting recombinant DNA molecule is then inserted into any of a variety of plasmid or phage expression vectors, which enable expression of the fusion protein molecule in an appropriate eukaryotic or prokaryotic host cell. BLYS fusion polypeptides may be generated by synthetic or semi-synthetic procedures as well.

The terms "BLYS target" or "BLYS target protein" are sometimes used herein and encompass BLYS and/or BLYS-like polypeptides. Thus, the BLYS binding polypeptides of the invention bind "BLYS target proteins" and can be used to bind, detect, remove, and/or purify "BLYS target proteins."

The term "binding polypeptide" is used herein to refer to any polypeptide capable of forming a binding complex with another molecule, polypeptide, peptidomimetic or transformant.

A "BLYS binding polypeptide" is a molecule of the invention that can bind BLYS target protein. Non-limiting examples of BLYS binding polypeptides of the invention are the polypeptide molecules having an amino acid sequence described herein (see SEQ ID NOs:1-12, 20-172, and 186-444). The term BLYS binding polypeptide also encompasses BLYS binding fragments and variants (including derivatives) of polypeptides having the specific amino acid sequences described herein (SEQ ID NOs:1-12, 20-172, and 186-444). By "variant" of an amino acid sequence as described herein is meant a polypeptide that binds BLYS, but does not necessarily comprise an identical or similar amino acid sequence of a BLYS binding polypeptide specified herein. BLYS binding polypeptides of the invention which are variants of a BLYS binding polypeptide specified herein satisfy at least one of the following: (a) a polypeptide comprising, or alternatively consisting of, an amino acid sequence that is at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% least 99%, or 100% identical to the amino acid sequence of a BLYS binding polypeptide sequence disclosed herein (SEQ ID NOs:1-12, 20-172, and 186-444), (b) a polypeptide encoded by a nucleotide sequence, the

complementary sequence of which hybridizes under stringent conditions to a nucleotide sequence encoding a BLyS binding polypeptide disclosed herein (e.g., a nucleic acid sequence encoding the amino acid sequence of SEQ ID NOs:1-12, 20-172, and 186-444), and/or a fragment of a BLyS binding polypeptide disclosed herein, of at least 5 amino acid residues, at least 10 amino acid residues, at least 15 amino acid residues, or at least 20 amino acid residues. BLyS binding polypeptides of the invention also encompass polypeptide sequences that have been modified for various applications provided that such modifications do not eliminate the ability to bind a BLyS target. Specific, non-limiting examples of modifications contemplated include C-terminal or N-terminal amino acid substitutions or peptide chain elongations for the purpose of linking the BLyS binder to a chromatographic material or other solid support. Other substitutions contemplated herein include substitution of one or both of a pair of cysteine residues that normally form disulfide links, for example with non-naturally occurring amino acid residues having reactive side chains, for the purpose of forming a more stable bond between those amino acid positions than the former disulfide bond. All such modified binding polypeptides are also considered BLyS binding polypeptides according to this invention so long as the modified polypeptides retain the ability to bind BLyS and/or BLyS-like polypeptides, and therefore, may be used in one or more of the various methods described herein, such as, to detect, purify, or isolate BLyS or BLyS-like polypeptides in or from a solution. BLyS binding polypeptides of the invention also include variants of the specific BLyS binding polypeptide sequences disclosed herein (e.g., SEQ ID NOs:1-12, 20-172, and 186-444) which have an amino acid sequence corresponding to one of these polypeptide sequences, but in which the polypeptide sequence is altered by substitutions, additions or deletions that provide for molecules that bind BLyS. Thus, the BLyS binding polypeptides include polypeptides containing, as a primary amino acid sequence, all or part of the particular BLyS binding polypeptide sequence including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence, resulting in a peptide which is functionally active. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Conservative substitutions for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral

amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such BLYS binding polypeptides can be made either by chemical peptide synthesis or by recombinant production from a nucleic acid encoding the BLYS binding polypeptide which nucleic acid has been mutated. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, in vitro site-directed mutagenesis (Hutchinson et al., *J. Biol. Chem.*, 253:6551 (1978)), use of TAB.RTM. linkers (Pharmacia), etc.

As used and understood herein, percent homology or percent identity of two amino acid sequences or of two nucleic acid sequences is determined using the algorithm of Karlin and Atschul (*Proc. Natl. Acad. Sci. USA*, 87: 2264-2268 (1990)), modified as in Karlin and Altschul (*Proc. Natl. Acad. Sci. USA*, 90: 5873-5877 (1993)). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (*J. Mol. Biol.*, 215: 403-410 (1990)). BLAST nucleotide searches are performed with the NBLAST program to obtain nucleotide sequences homologous to a nucleic acid molecule described herein. BLAST protein searches are performed with the XBLAST program to obtain amino acid sequences homologous to a reference polypeptide. To obtain gapped alignments for comparison purposes, Gapped BLAST is utilized as described in Altschul et al. (*Nucleic Acids Res.*, 25: 3389-3402 (1997)). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) are used. See, <http://www.ncbi.nlm.nih.gov>. Alternatively, the percent identity of two amino acid sequences or of two nucleic acid sequences can be determined once the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino acid or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide at the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = number of identical overlapping positions/total number of positions  $\times$  100%). In one embodiment, the two sequences are the same length.

The term "polypeptide", as used herein, refers to a linear, branched, or cyclic (e.g., containing a loop structure) polymer of two or more amino acid residues linked with a peptide bond. The term "polypeptide" is not restricted to any particular upper limit of amino acid residues. Thus, the BLYS affinity ligands of the invention that comprise an amino acid sequence described herein are properly referred to as "BLYS binding polypeptides" because such binding polypeptides contain at least two amino acid residues held together by a peptide bond, even though such molecules may also contain one or more additional moieties or groups that are not amino acids, such as N-terminal and/or C-terminal capping or functional groups, and that may or may not be involved in a peptide bond. The polypeptides of the invention may be monovalent, divalent, trivalent, or multivalent and may comprise one or more of the BLYS binding polypeptides having the amino acid sequence of SEQ ID NOs:1-12, 20-172, and 186-444 and/or fragments or variants thereof. The term "peptide" is used herein to have the same meaning as "polypeptide."

"Feed stream": BLYS and BLYS-like polypeptides that are bound by a BLYS binding polypeptide of this invention may be produced by any method known in the art, including, but not limited to, chemical synthesis; production in transformed host cells; secretion into culture medium by naturally occurring cells or recombinantly transformed bacteria, yeasts, fungi, insect cells, plant cells, and mammalian cells; production in genetically engineered organisms (for example, transgenic mammals); and production in non-genetically engineered organisms. The solution, sample, or mixture that contains a BLYS or BLYS-like polypeptide as it is produced or is found present in a production solution will sometimes be referred to as the "feed stream".

The term "binding" refers to the determination by standard techniques that a binding polypeptide recognizes and binds to a given target. Such standard techniques include, but are not limited to, affinity chromatography, equilibrium dialysis, gel filtration, enzyme linked immunosorbent assay (ELISA), FACS analysis, and the monitoring of spectroscopic changes that result from binding, e.g., using fluorescence anisotropy, either by direct binding measurements or competition assays with another binder.

The term "specificity" refers to a binding polypeptide of the invention that has a higher binding affinity for one target over another. Thus, the term "BLYS target protein specificity" refers to a molecule having a higher affinity for BLYS target protein as compared with another molecule that is not a BLYS target protein.

Other terms are defined as necessary in the text below.



## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel binding moieties for BLyS. Such binding moieties make possible the efficient detection and isolation of BLyS or BLyS-like polypeptides in tissues or in a solution or system that contains BLyS or BLyS-like polypeptides. The BLyS binding polypeptides disclosed herein can also be used to immobilize BLyS targets and provide a means of removing BLyS target proteins from solutions or systems containing them. The preferred binding moieties of the present invention bind BLyS with high affinity, i.e., acting at low concentrations.

### BLyS Binding Polypeptides

The present invention provides new polypeptides and families of polypeptides that specifically bind to B lymphocyte stimulator protein (BLyS) and/or BLyS-like polypeptides. In particular, the invention encompasses polypeptides that specifically bind to a polypeptide or polypeptide fragment of human BLyS (SEQ ID NOs:173 and/or 174) or BLyS expressed on human monocytes; murine BLyS (SEQ ID NOs:175 and/or 176) or BLyS expressed on murine monocytes; rat BLyS (either the soluble forms as given in SEQ ID NOs:177, 178, 179 and/or 180 or in a membrane associated form, e.g., on the surface of rat monocytes); or monkey BLyS (e.g., the monkey BLyS polypeptides of SEQ ID NOS:181 and/or 182, the soluble form of monkey BLyS, or BLyS expressed on monkey monocytes); preferably human BLyS.

In specific preferred embodiments, the BLyS binding polypeptides of the invention bind BLyS and/or BLyS-like polypeptides with high affinity. In other embodiments, the BLyS binding polypeptides of the invention reversibly bind BLyS and/or BLyS-like polypeptides. In still other embodiments, the BLyS binding polypeptides of the invention irreversibly bind BLyS and/or BLyS-like polypeptides.

The cysteine residues in polypeptides are believed to form a disulfide bond, which would cause the polypeptide containing these cysteine residues to form a stable loop structure under non-reducing conditions. Especially preferred BLyS binding polypeptides of the invention are polypeptide molecules that comprise amino acid sequences that form stable loop structures or other stable structures that bind BLyS or BLyS-like polypeptides.

In specific embodiments, the invention relates to BLyS binding polypeptides comprising, or alternatively consisting of, an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-12, 20-172, and 186-444, preferably SEQ ID NOs:163-172 or 436-444 as referred to above and in Tables 1-8, 14 and 15 and in Examples 2, 5 and 6 below. Analysis of the sequences of the BLyS binding polypeptides isolated as described herein shows a strong selection for polypeptides containing the tetrapeptide Asp-Xaa-Leu-Thr (SEQ ID NO:446), and therefore in its broadest aspects, the present invention relates to polypeptides capable of binding to BLyS comprising the polypeptide Asp-Xaa-Leu-Thr (SEQ ID NO:446), where Xaa is Pro, Ser, Thr, Phe, Leu, Tyr, Cys, or Ala (preferably Pro or Ser).

Seven consensus sequences (SEQ ID NOs:1-7) have been determined based on the specific BLyS binding polypeptides shown in Tables 1-8. In specific embodiments, BLyS binding polypeptides of the invention comprise one or more of these sequences. Such preferred BLyS binding polypeptides include polypeptides with the potential to form a cyclic or loop structure between invariant Cys residues comprising, or alternatively consisting of, an amino acid sequence selected from A-E (SEQ ID NOs:1-5):

(A)  $X_1-X_2-X_3-Cys-X_5-Phe-X_7-Trp-Glu-Cys-X_{11}-X_{12}-X_{13}$  (SEQ ID NO:1),

wherein

$X_1$  is Ala, Asn, Lys, or Ser;

$X_2$  is Ala, Glu, Met, Ser, or Val;

$X_3$  is Ala, Asn, Lys, or Pro (preferably Lys);

$X_5$  is Phe, Trp, or Tyr (preferably Tyr);

$X_7$  is Pro or Tyr (preferably Pro);

$X_{11}$  is Ala, Gln, His, Phe, or Val;

$X_{12}$  is Asn, Gln, Gly, His, Ser, or Val; and

$X_{13}$  is Ala, Asn, Gly, Ile, Pro, or Ser,

wherein said polypeptide binds BLyS and/or BLyS-like polypeptides; or

(B)  $X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-Cys-X_{12}-X_{13}-X_{14}$  (SEQ ID NO:2),

wherein

$X_1$  is Ala, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, or is absent;

$X_2$  is Ala, Asn, Asp, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X<sub>3</sub> is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Asp);

X<sub>5</sub> is Asp, Ile, Leu, or Tyr (preferably Asp or Leu);

X<sub>6</sub> is Arg, Asp, Glu, His, Ile, Leu, Lys, Phe, Pro, Tyr, or Val (preferably Glu or Leu);

5 X<sub>7</sub> is His, Leu, Lys, or Phe (preferably His or Leu);

X<sub>8</sub> is Leu, Pro, or Thr (preferably Thr or Pro);

X<sub>9</sub> is Arg, Asn, Gly, His, Ile, Lys, Met, or Trp (preferably Lys);

X<sub>10</sub> is Ala, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Ser, Trp, Tyr, or Val;

X<sub>12</sub> is Asp, Gln, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Trp, Tyr, or Val;

10 X<sub>13</sub> is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val; and

X<sub>14</sub> is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Trp, Tyr, Val, or is absent, wherein said polypeptide binds BLYS and/or BLYS-like polypeptides; or

(C) X<sub>1</sub>-X<sub>2</sub>-X<sub>3</sub>-Cys-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-X<sub>10</sub>-X<sub>11</sub>-Cys-X<sub>13</sub>-X<sub>14</sub>-X<sub>15</sub> (SEQ ID NO:3),

wherein

X<sub>1</sub> is Ala, Arg, Asn, Asp, Leu, Lys, Phe, Pro, Ser, or Thr;

X<sub>2</sub> is Asn, Asp, Gln, His, Ile, Lys, Pro, Thr, or Trp;

X<sub>3</sub> is Ala, Arg, Asn, Gln, Glu, His, Phe, Pro, or Thr (preferably Ala);

X<sub>5</sub> is Asn, Asp, Pro, Ser, or Thr (preferably Asp);

20 X<sub>6</sub> is Arg, Asp, Ile, Leu, Met, Pro, or Val (preferably Ile);

X<sub>7</sub> is Ala, Ile, Leu, Pro, Thr, or Val (preferably Val or Leu);

X<sub>8</sub> is Asn, His, Ile, Leu, Lys, Phe, or Thr (preferably Thr);

X<sub>9</sub> is Asn, Glu, Gly, His, Leu, Lys, Met, Pro, or Thr (preferably Leu);

X<sub>10</sub> is Arg, Asn, Asp, Gln, Glu, Gly, Ile, Lys, Met, Pro, Ser, or Trp;

25 X<sub>11</sub> is Arg, Glu, Gly, Lys, Phe, Ser, Trp, or Tyr (preferably Ser);

X<sub>13</sub> is Gln, Glu, Ile, Leu, Phe, Pro, Ser, Tyr, or Val (preferably Val);

X<sub>14</sub> is Asn, Gly, Ile, Phe, Pro, Thr, Trp, or Tyr; and

X<sub>15</sub> is Asn, Asp, Glu, Leu, Lys, Met, Pro, or Thr (preferably Glu or Pro),

wherein said polypeptide binds BLYS and/or BLYS-like polypeptides; or

30 (D) X<sub>1</sub>-X<sub>2</sub>-X<sub>3</sub>-Cys-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-X<sub>10</sub>-X<sub>11</sub>-X<sub>12</sub>-Cys-X<sub>14</sub>-X<sub>15</sub>-X<sub>16</sub> (SEQ ID NO:4),

wherein

X<sub>1</sub> is Asn, Asp, His, Leu, Phe, Pro, Ser, Tyr, or is absent (preferably Ser);

X<sub>2</sub> is Arg, Asn, Asp, His, Phe, Ser, or Trp (preferably Arg);

X<sub>3</sub> is Asn, Asp, Leu, Pro, Ser, or Val (preferably Asn or Asp);

5 X<sub>5</sub> is Asp, Gln, His, Ile, Leu, Lys, Met, Phe, or Thr;

X<sub>6</sub> is His, Ile, Leu, Met, Phe, Pro, Trp, or Tyr;

X<sub>7</sub> is Asp, His, Leu, or Ser (preferably Asp);

X<sub>8</sub> is Ala, Arg, Asp, Glu, Leu, Phe, Pro, or Thr (preferably Glu or Pro);

X<sub>9</sub> is Ala, Arg, Asn, or Leu (preferably Leu);

10 X<sub>10</sub> is Ile, Leu, Met, Pro, Ser, or Thr (preferably Thr);

X<sub>11</sub> is Ala, Arg, Asn, Gly, His, Lys, Ser, or Tyr;

X<sub>12</sub> is Ala, Arg, Asn, Gln, Leu, Met, Ser, Trp, Tyr, or Val;

X<sub>14</sub> is Asp, Gly, Leu, Phe, Tyr, or Val (preferably Leu);

X<sub>15</sub> is Asn, His, Leu, Pro, or Tyr (preferably His, Leu or Pro); and

X<sub>16</sub> is Asn, Asp, His, Phe, Ser, or Tyr, (preferably Asp or Ser),

wherein said polypeptide binds BLYS and/or BLYS-like polypeptides; or

(E) X<sub>1</sub>-X<sub>2</sub>-X<sub>3</sub>-Cys-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-X<sub>10</sub>-X<sub>11</sub>-X<sub>12</sub>-X<sub>13</sub>-X<sub>14</sub>-Cys-X<sub>16</sub>-X<sub>17</sub>-X<sub>18</sub>

(SEQ ID NO:5),

wherein

20 X<sub>1</sub> is Arg, Asp, Gly, His, Leu, Phe, Pro, Ser, Trp, Tyr, or is absent (preferably Arg);

X<sub>2</sub> is Ala, Arg, Asn, Asp, Gly, Pro, Ser, or is absent (preferably Asn, Asp, Gly, or Pro);

X<sub>3</sub> is Arg, Asn, Gln, Glu, Gly, Lys, Met, Pro, Trp or Val (preferably Gly or Met);

X<sub>5</sub> is Arg, Asn, Gln, Glu, His, Leu, Phe, Pro, Trp, Tyr, or Val (preferably Trp, Tyr, or Val);

X<sub>6</sub> is Arg, Asp, Gln, Gly, Ile, Lys, Phe, Thr, Trp or Tyr (preferably Asp);

25 X<sub>7</sub> is Ala, Arg, Asp, Glu, Gly, Leu, Ser, or Tyr (preferably Asp);

X<sub>8</sub> is Asp, Gln, Glu, Leu, Met, Phe, Pro, Ser, or Tyr (preferably Leu);

X<sub>9</sub> is Asp, Leu, Pro, Thr, or Val (preferably Leu or Thr);

X<sub>10</sub> is Arg, Gln, His, Ile, Leu, Lys, Met, Phe, Thr, Trp or Tyr (preferably Lys or Thr);

X<sub>11</sub> is Ala, Arg, Asn, Gln, Glu, His, Leu, Lys, Met, or Thr (preferably Arg or Leu);

30 X<sub>12</sub> is Ala, Asn, Gln, Gly, Leu, Lys, Phe, Pro, Thr, Trp, or Tyr (preferably Thr or Trp);

X<sub>13</sub> is Ala, Arg, Gln, His, Lys, Met, Phe, Pro, Thr, Trp, or Tyr (preferably Met or Phe);

X<sub>14</sub> is Arg, Gln, Glu, Gly, His, Leu, Met, Phe, Pro, Ser, Thr, Tyr, or Val (preferably Val);  
X<sub>16</sub> is Arg, Asp, Gly, His, Lys, Met, Phe, Pro, Ser, or Trp (preferably Met);  
X<sub>17</sub> is Arg, Asn, Asp, Gly, His, Phe, Pro, Ser, Trp or Tyr, (preferably Arg, His, or Tyr); and  
X<sub>18</sub> is Ala, Arg, Asn, Asp, His, Leu, Phe, or Trp (preferably His or Asn),  
5 wherein said polypeptide binds BLYS and/or BLYS-like polypeptides.

Additional preferred embodiments include linear polypeptides comprising, or alternatively consisting of, an amino acid sequence selected from F and G (SEQ ID NOs:6 and 7):

(F) X<sub>1</sub>-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-X<sub>10</sub>-X<sub>11</sub>-X<sub>12</sub> (SEQ ID NO:6),

10 wherein

X<sub>1</sub> is Ala, Arg, Gly, His, Leu, Lys, Met, Phe, Trp, Tyr, or Val (preferably Gly, Tyr, or Val);  
X<sub>2</sub> is Ala, Arg, Gln, His, Ile, Leu, Phe, Thr, Trp, or Tyr (preferably His or Tyr);  
X<sub>3</sub> is Ala, Asp, Lys, Phe, Thr, Trp or Tyr (preferably Asp or Tyr);  
X<sub>4</sub> is Arg, Asp, Gln, Lys, Met, Phe, Pro, Ser, Tyr, or Val (preferably Asp or Gln);  
X<sub>5</sub> is Asp, Leu, Lys, Phe, Pro, Ser, or Val (preferably Leu or Ser);  
X<sub>6</sub> is His, Ile, Leu, Pro, Ser, or Thr (preferably Leu or Thr);  
X<sub>7</sub> is Arg, Gly, His, Leu, Lys, Met, or Thr (preferably Lys or Thr);  
X<sub>8</sub> is Ala, Arg, Asn, Ile, Leu, Lys, Met, or Thr (preferably Leu or Lys);  
X<sub>9</sub> is Ala, Asn, Arg, Asp, Glu, Gly, His, Leu, Met, Ser, Trp, Tyr, or Val (preferably Met or Ser);  
X<sub>10</sub> is Ile, Leu, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Thr or Leu);  
X<sub>11</sub> is Ala, Arg, Gly, His, Ile, Leu, Lys, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Pro or Thr);  
and

X<sub>12</sub> is Arg, Asp, His, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Arg or Pro),  
wherein said polypeptide binds BLYS and/or BLYS-like polypeptides; or

25 (G) X<sub>1</sub>-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-X<sub>10</sub>-X<sub>11</sub>-X<sub>12</sub>-X<sub>13</sub> (SEQ ID NO:7),

wherein

X<sub>1</sub> is Asp, Gln, Glu, Gly, His, Lys, Met, or Trp (preferably Glu, Lys);  
X<sub>2</sub> is Arg, Gln, His, Ile, Leu, or Pro (preferably His or Pro);  
X<sub>3</sub> is Asp, Gly, Ile, Lys, Thr, Tyr or Val (preferably Tyr);  
30 X<sub>4</sub> is Asn, Asp, Gln, Glu, Met, Pro, Ser, or Tyr (preferably Asp or Gln);  
X<sub>5</sub> is Asn, Asp, His, Ile, Leu, Met, Pro, Thr or Val (preferably Asn or Thr);

X<sub>6</sub> is Asp, Glu, His, Leu, Lys, Pro, or Val (preferably Asp or Pro);

X<sub>7</sub> is Arg, Asn, Gln, His, Ile, Leu, Met, Pro, or Thr (preferably Ile or Pro);

X<sub>8</sub> is Gln, Gly, His, Leu, Met, Ser, or Thr (preferably Leu or Thr);

X<sub>9</sub> is Asn, Gln, Gly, His, Leu, Lys, Ser, or Thr (preferably Lys);

5 X<sub>10</sub> is Ala, Gly, Ile, Leu, Lys, Met, or Phe (preferably Gly or Met);

X<sub>11</sub> is Ala, Glu, His, Ile, Leu, Met, Ser, Thr, Trp, Tyr, or Val (preferably Ala or Thr);

X<sub>12</sub> is Arg, Gln, Glu, Gly, His, Ile, Lys, Tyr, or Val (preferably Arg or His); and

X<sub>13</sub> is Arg, Asn, Glu, His, Ile, Ser, Thr, Trp, or Val (preferably His),

wherein said polypeptide binds BLYS and/or BLYS-like polypeptides.

10 Said polypeptides may have additional amino acids attached at either or both of the N- and C-terminal ends.

Examination of the sequence information and binding data from the isolates of libraries containing polypeptides with the potential to form loop structures (i.e., libraries designated TN6, TN7, TN8, TN9, TN10 and TN12) identifies a series of BLYS binding polypeptides that may form loop structures. In specific embodiments, BLYS binding polypeptides of the invention comprise, or alternatively consist of, an amino acid sequence selected from H-L (SEQ ID NOs:8-12):

(H) Cys-X<sub>2</sub>-Phe-X<sub>4</sub>-Trp-Glu-Cys (SEQ ID NO:8),

wherein

20 X<sub>2</sub> is Phe, Trp, or Tyr (preferably Tyr); and

X<sub>4</sub> is Pro or Tyr (preferably Pro); or

(I) Cys-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-Cys (SEQ ID NO:9),

wherein

X<sub>2</sub> is Asp, Ile, Leu, or Tyr (preferably Asp or Leu);

25 X<sub>3</sub> is Arg, Asp, Glu, His, Ile, Leu, Lys, Phe, Pro, Tyr, or Val (preferably Glu or Leu);

X<sub>4</sub> is His, Leu, Lys, or Phe (preferably His or Leu);

X<sub>5</sub> is Leu, Pro, or Thr (preferably Thr or Pro);

X<sub>6</sub> is Arg, Asn, Gly, His, Ile, Lys, Met, or Trp (preferably Lys); and

X<sub>7</sub> is Ala, Asn, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Ser, Trp, Tyr, or Val; or

30 (J) Cys-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-Cys (SEQ ID NO:10),

wherein

X<sub>2</sub> is Asn, Asp, Pro, Ser, or Thr (preferably Asp);

X<sub>3</sub> is Arg, Asp, Ile, Leu, Met, Pro, or Val (preferably Ile);

X<sub>4</sub> is Ala, Ile, Leu, Pro, Thr, or Val (preferably Val or Leu);

X<sub>5</sub> is Asn, His, Ile, Leu, Lys, Phe, or Thr (preferably Thr);

5 X<sub>6</sub> is Asn, Glu, Gly, His, Leu, Lys, Met, Pro, or Thr (preferably Leu);

X<sub>7</sub> is Arg, Asn, Asp, Gln, Glu, Gly, Ile, Lys, Met, Pro, Ser, or Trp;

X<sub>8</sub> is Arg, Glu, Gly, Lys, Phe, Ser, Trp, or Tyr (preferably Ser); or

(K) Cys-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-Cys (SEQ ID NO:11),

wherein

10 X<sub>2</sub> is Asp, Gln, His, Ile, Leu, Lys, Met, Phe, or Thr;

X<sub>3</sub> is His, Ile, Leu, Met, Phe, Pro, Trp, or Tyr;

X<sub>4</sub> is Asp, His, Leu, or Ser (preferably Asp);

X<sub>5</sub> is Ala, Arg, Asp, Glu, Leu, Phe, Pro, or Thr (preferably Glu or Pro);

X<sub>6</sub> is Ala, Arg, Asn, or Leu (preferably Leu);

X<sub>7</sub> is Ile, Leu, Met, Pro, Ser, or Thr (preferably Thr);

X<sub>8</sub> is Ala, Arg, Asn, Gly, His, Lys, Ser, or Tyr;

X<sub>9</sub> is Ala, Arg, Asn, Gln, Leu, Met, Ser, Trp, Tyr, or Val; or

(L) Cys-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-X<sub>5</sub>-X<sub>6</sub>-X<sub>7</sub>-X<sub>8</sub>-X<sub>9</sub>-X<sub>10</sub>-X<sub>11</sub>-Cys (SEQ ID NO: 12),

wherein

20 X<sub>2</sub> is Arg, Asn, Gln, Glu, His, Leu, Phe, Pro, Trp, Tyr, or Val (preferably Trp, Tyr, or Val);

X<sub>3</sub> is Arg, Asp, Gln, Gly, Ile, Lys, Phe, Thr, Trp or Tyr (preferably Asp);

X<sub>4</sub> is Ala, Arg, Asp, Glu, Gly, Leu, Ser, or Tyr (preferably Asp);

X<sub>5</sub> is Asp, Gln, Glu, Leu, Met, Phe, Pro, Ser, or Tyr (preferably Leu);

X<sub>6</sub> is Asp, Leu, Pro, Thr, or Val (preferably Leu or Thr);

25 X<sub>7</sub> is Arg, Gln, His, Ile, Leu, Lys, Met, Phe, Thr, Trp or Tyr (preferably Lys or Thr);

X<sub>8</sub> is Ala, Arg, Asn, Gln, Glu, His, Leu, Lys, Met, or Thr (preferably Arg or Leu);

X<sub>9</sub> is Ala, Asn, Gln, Gly, Leu, Lys, Phe, Pro, Thr, Trp, or Tyr (preferably Thr or Trp);

X<sub>10</sub> is Ala, Arg, Gln, His, Lys, Met, Phe, Pro, Thr, Trp, or Tyr (preferably Met or Phe);

X<sub>11</sub> is Arg, Gln, Glu, Gly, His, Leu, Met, Phe, Pro, Ser, Thr, Tyr, or Val (preferably Val);

30 wherein said polypeptides bind BLYS and/or BLYS-like polypeptides.

In additional preferred embodiments of the present invention, BLYS binding polypeptides comprise the following amino acid sequence M (SEQ ID NO:447):

(M) Ala-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-Asp-X<sub>6</sub>-Leu-Thr-X<sub>9</sub>-Leu-X<sub>11</sub>-X<sub>12</sub>-X<sub>13</sub>-X<sub>14</sub> (SEQ ID NO:447),

wherein

X<sub>2</sub> is Asn, Ser, Tyr, Asp, Phe, Ile, Gln, His, Pro, Lys, Leu, Met, Thr, Val, Glu, Ala, Gly, Cys, or Trp (i.e., any amino acid except Arg; preferably Asn);

X<sub>3</sub> is Trp, Glu, Lys, Cys, Leu, Ala, Arg, Gly, or Ser (preferably Trp);

X<sub>4</sub> is Tyr, Phe, Glu, Cys, Asn (preferably Tyr);

X<sub>6</sub> is Pro, Ser, Thr, Phe, Leu, Tyr, Cys, or Ala (preferably Pro or Ser);

X<sub>9</sub> is Lys, Asn, Gln, Gly, or Arg (preferably Lys);

X<sub>11</sub> is Trp, Ser, Thr, Arg, Cys, Tyr, or Lys (preferably Trp);

X<sub>12</sub> is Leu, Phe, Val, Ile, or His (preferably Leu);

X<sub>13</sub> is Pro, Leu, His, Ser, Arg, Asn, Gln, Thr, Val, Ala, Cys, Ile, Phe, or Tyr (i.e., not Asp, Glu, Gly, Lys, Met, or Trp; preferably Pro); and

X<sub>14</sub> is Asp, Glu, Asn, Val, His, Gln, Arg, Gly, Ser, Tyr, Ala, Cys, Lys, Ile, Thr or Leu (i.e., not Phe, Met, Pro, or Trp; preferably Asp, Val or Glu).

Preferred embodiments are polypeptides comprising a core sequence of the formula N:

(N) X<sub>1</sub>-X<sub>2</sub>-Asp-X<sub>4</sub>-Leu-Thr-X<sub>7</sub>-Leu-X<sub>9</sub>-X<sub>10</sub> (SEQ ID NO:448),

wherein

X<sub>1</sub> is Trp, Glu, Lys, Cys, Leu, Ala, Arg, Gly, or Ser (preferably Trp);

X<sub>2</sub> is Tyr, Phe, Glu, Cys, Asn (preferably Tyr);

X<sub>4</sub> is Pro, Ser, Thr, Phe, Leu, Tyr, Cys, or Ala (preferably Pro or Ser);

X<sub>7</sub> is Lys, Asn, Gln, Gly, or Arg (preferably Lys);

X<sub>9</sub> is Trp, Ser, Thr, Arg, Cys, Tyr, or Lys (preferably Trp); and

X<sub>10</sub> is Leu, Phe, Val, Ile, or His (preferably Leu).

Especially preferred BLYS binding polypeptides according to the present invention comprise the core peptide Trp-Tyr-Asp-Pro-Leu-Thr-Lys-Leu-Trp-Leu (SEQ ID NO:436).

The BLYS binding polypeptides described above may have additional amino acids attached at either or both of the N- and C-terminal ends.



BLyS binding polypeptide molecules of the invention may also have an amino terminal (N-terminal) capping or functional group, such as an acetyl group, which, for example, blocks the amino terminal amino group from undesirable reactions or is useful in linking the BLyS binding polypeptide to another molecule, matrix, resin, or solid support. BLyS binding polypeptides of the invention may also have a carboxy terminal (C-terminal) capping or functional group, such as an amide group, which, for example, blocks the C-terminal carboxyl group from undesirable reactions or provides a functional group useful in conjugating the binding polypeptide to other molecules, matrices, resins, or solid supports. Preferably, the N- and/or C-terminal capping groups are polypeptide linker molecules. An especially preferred C-terminal linker molecule that is useful for immobilizing a BLyS binding polypeptide of the invention to a solid support or chromatographic matrix material comprises the amino acid sequence Pro-Gly-Pro-Glu-Gly-Gly-Gly-Lys (SEQ ID NO:13). Another useful C-terminal linker, e.g., for fluoresceinating peptides, is Gly-Gly-Lys (see Table 15).

The invention also encompasses, BLyS binding polypeptides that have been modified, for example, to increase or decrease the stability of the molecule, while retaining the ability to bind BLyS and/or BLyS-like polypeptides. An example of a modified BLyS binding polypeptide of the invention is a polypeptide in which one of two cysteine residues is substituted with a non-naturally occurring amino acid that is capable of condensing with the remaining cysteine side chain to form a stable thioether bridge, thereby generating a cyclic BLyS binding polypeptide. Such cyclic thioether molecules of synthetic peptides may be routinely generated using techniques known in the art and described, e.g., in PCT publication WO 97/46251, incorporated herein by reference.

In another embodiment, the invention provides BLyS binding polypeptides of the invention attached, coupled, linked or adhered to a matrix or resin or solid support. Techniques for attaching linking or adhering polypeptides to matrices, resins and solid supports are well known in the art. Suitable matrices, resins or solid supports for these materials may be any composition known in the art to which binding polypeptides are commonly attached, coupled, linked, or adhered, including but not limited to, a chromatographic resin or matrix, such as SEPHAROSE-4 FF agarose beads, the wall or floor of a well in a plastic microtiter dish, such as used in an enzyme-linked immunosorbent assay (ELISA), or a silica based biochip. Materials useful as solid supports on which to immobilize binding polypeptides of the invention include,

but are not limited to, polyacrylamide, agarose, silica, nitrocellulose, paper, plastic, nylon, metal, and combinations thereof. A BLYS binding polypeptide of the invention may be immobilized on a matrix, resin or solid support material by a non-covalent association or by covalent bonding, using techniques known in the art. Preferably, a BLYS binding polypeptide of the invention is  
5 immobilized on SEPHAROSE-4 FF agarose chromatographic material. More preferably, a BLYS binding polypeptide of the invention is coupled to a chromatography material such as SEPHAROSE-4FF (agarose). In an even more preferred embodiment, a BLYS binding polypeptide of the invention is coupled to a chromatography material using a linker molecule. A preferred linker molecule according to the present invention is a polypeptide comprising the  
10 amino acid sequence Pro-Gly-Pro-Glu-Gly-Gly-Gly-Lys (SEQ ID NO:13). Most preferably, the affinity chromatography material of the invention comprises a BLYS binding polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:163-172, which is linked to a chromatography material by a polypeptide linker molecule having the amino acid sequence Pro-Gly-Pro-Glu-Gly-Gly-Gly-Lys (SEQ ID NO:13). BLYS binding polypeptides of the invention attached, coupled, linked or adhered to a matrix or resin or solid support are useful for methods of detecting, isolating and purifying BLYS and/or BLYS like polypeptides, particularly for purification of BLYS and/or BLYS like polypeptides by affinity chromatography.

In certain preferred embodiments, the BLYS binding polypeptides of the present invention or phage displaying such binding polypeptides, irreversibly bind the BLYS protein in its native, soluble trimeric form.

In certain preferred embodiments, the BLYS binding polypeptides of the present invention or phage displaying such binding polypeptides, reversibly bind the BLYS protein in its native, soluble trimeric form.

BLYS binding polypeptides of the invention bind BLYS target protein with high affinity.  
25 In specific embodiments, BLYS binding polypeptides of the invention bind BLYS target proteins with a dissociation constant or  $K_D$  of less than or equal to  $5 \times 10^{-2}$  M,  $10^{-2}$  M,  $5 \times 10^{-3}$  M,  $10^{-3}$  M,  $5 \times 10^{-4}$  M,  $10^{-4}$  M,  $5 \times 10^{-5}$  M, or  $10^{-5}$  M. More preferably, BLYS binding polypeptides of the invention bind BLYS target proteins with a dissociation constant or  $K_D$  less than or equal to  $5 \times 10^{-6}$  M,  $10^{-6}$  M,  $5 \times 10^{-7}$  M,  $10^{-7}$  M,  $5 \times 10^{-8}$  M, or  $10^{-8}$  M. Even more preferably, BLYS binding  
30 polypeptides of the invention bind BLYS target proteins with a dissociation constant or  $K_D$  less

than or equal to  $5 \times 10^{-9}$  M,  $10^{-9}$  M,  $5 \times 10^{-10}$  M,  $10^{-10}$  M,  $5 \times 10^{-11}$  M,  $10^{-11}$  M,  $5 \times 10^{-12}$  M,  $10^{-12}$  M,  $5 \times 10^{-13}$  M,  $10^{-13}$  M,  $5 \times 10^{-14}$  M,  $10^{-14}$  M,  $5 \times 10^{-15}$  M, or  $10^{-15}$  M.

In certain preferred embodiments, BLyS binding polypeptides of the invention reversibly bind BLyS and/or BLyS-like polypeptides and release bound BLyS protein in an active form, preferably in the native soluble trimeric form, under specific release conditions. In specific embodiments, BLyS binding polypeptides of the invention bind BLyS target proteins with off-rates or  $k_{\text{off}}$  greater than or equal to  $10^{-10} \text{ s}^{-1}$ ,  $5 \times 10^{-9} \text{ s}^{-1}$ ,  $10^{-9} \text{ s}^{-1}$ ,  $5 \times 10^{-8} \text{ s}^{-1}$ ,  $10^{-8} \text{ s}^{-1}$ ,  $5 \times 10^{-7} \text{ s}^{-1}$ ,  $10^{-7} \text{ s}^{-1}$ ,  $5 \times 10^{-6} \text{ s}^{-1}$ ,  $10^{-6} \text{ s}^{-1}$ ,  $5 \times 10^{-5} \text{ s}^{-1}$ ,  $10^{-5} \text{ s}^{-1}$ ,  $5 \times 10^{-4} \text{ s}^{-1}$ ,  $10^{-4} \text{ s}^{-1}$ ,  $5 \times 10^{-3} \text{ s}^{-1}$ ,  $10^{-3} \text{ s}^{-1}$ ,  $5 \times 10^{-2} \text{ s}^{-1}$ ,  $10^{-2} \text{ s}^{-1}$ ,  $5 \times 10^{-1} \text{ s}^{-1}$ , or  $10^{-1} \text{ s}^{-1}$ .

Binding experiments to determine  $K_D$  and off-rates can be performed in a number of conditions including, but not limited to, [pH 6.0, 0.01% Tween 20], [pH 6.0, 0.1% gelatin], [pH5.0, 0.01% Tween 20], [pH9.0, 0.1% Tween 20], [pH6.0, 15% ethylene glycol, 0.01 % Tween20], [pH5.0, 15% ethylene glycol, 0.01 % Tween 20], and [pH9.0, 15% ethylene glycol, 0.01 % Tween 20] The buffers in which to make these solutions can readily be determined by one of skill in the art, and depend largely on the desired pH of the final solution. Low pH solutions (< pH 5.5) can be made, for example, in citrate buffer, glycine-HCl buffer, or in succinic acid buffer. High pH solutions can be made, for example, in Tris-HCl, phosphate buffers, or sodium bicarbonate buffers. A number of conditions may be used to determine  $K_D$  and off-rates for the purpose of determining, for example, optimal pH and/or salt concentrations.

In certain embodiments, BLyS binding polypeptides of the invention reversibly bind BLyS and/or BLyS-like polypeptides, preferably in the native soluble, trimeric form.

In preferred embodiments, BLyS binding polypeptides of the invention reversibly bind only the native soluble, trimeric form of BLyS.

In certain embodiments, BLyS binding polypeptides of the invention irreversibly bind BLyS and/or BLyS-like polypeptides, preferably in the native soluble, trimeric form.

In preferred embodiments, BLyS binding polypeptides of the invention irreversibly bind only the native soluble, trimeric form of BLyS.

In some screening or assay procedures, it is possible and more convenient to use recombinant bacteriophage that display a particular BLyS binding polypeptide instead of using isolated BLyS binding polypeptide. Such procedures include phage-based ELISA protocols and immobilization of phage displaying a binding polypeptide to chromatographic materials. Such

screening assays and procedures are routine in the art and may be readily adapted for procedures using the recombinant bacteriophage of the present invention.

The invention also encompasses BLYS binding polypeptides that competitively inhibit the binding of a BLYS binding polypeptide disclosed herein (e.g., a polypeptide having the amino acid sequence of SEQ ID NOS:163-168) for binding to BLYS. Competitive inhibition can be determined by any suitable method known in the art, for example, using the competitive binding assays described herein. In preferred embodiments, the polypeptide competitively inhibits the binding of a BLYS binding polypeptide disclosed herein (e.g., a polypeptide having the amino acid sequence of SEQ ID NOS:163-168) to BLYS by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50%. In a more preferred embodiment, the BLYS binding polypeptide competitively inhibits the binding of a BLYS binding polypeptide disclosed herein (e.g., a polypeptide having the amino acid sequence of SEQ ID NOS:163-168) to the native soluble trimeric form of BLYS, by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50%.

In a further embodiment, the present invention encompasses a composition of matter comprising isolated nucleic acids, preferably DNA, encoding a BLYS binding polypeptide of the invention. In a specific embodiment, nucleic acid molecules of the invention encode a BLYS binding polypeptide of the invention as provided in SEQ ID NOS:1-12, 20-172, and 186-444. In additional embodiments, nucleic acid molecules of the invention encode a polypeptide variant or fragment of a polypeptide having an amino acid sequence of SEQ ID NOS:1-12, 20-172, and 186-444. In a further additional embodiment, nucleic acid molecules of the invention encode a BLYS binding polypeptide, the complementary strand of which nucleic acid hybridizes to a polynucleotide sequence encoding a polypeptide described in Tables 1-8 and in Examples 2 and 5 (SEQ ID NOS:1-12, 20-172, and 186-444), under stringent conditions, e.g., hybridization to filter-bound DNA in 6x sodium chloride/sodium citrate (SSC) at about 45° C followed by one or more washes in 0.2xSSC/0.1% SDS at about 50-65° C, under highly stringent conditions, e.g., hybridization to filter-bound nucleic acid in 6xSSC at about 45° C followed by one or more washes in 0.1xSSC/0.2% SDS at about 68° C, or under other stringent hybridization conditions which are known to those of skill in the art (see, for example, Ausubel et al., eds. , 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York at pages 6.3.1-6.3.6 and 2.10.3).

The present invention also relates to recombinant vectors that include the isolated nucleic acid molecules encoding the BLYS binding polypeptides of the present invention (as well as fragments and variants thereof), and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells. The invention further provides for the use of such recombinant vectors in the production of BLYS binding polypeptides by recombinant techniques.

The BLYS binding polypeptides, nucleic acids, transformed host cells, and genetically engineered viruses and phage of the invention (e.g., recombinant phage), have uses that include, but are not limited to, the detection, isolation, and purification of BLYS.

In another embodiment of the invention, recombinant bacteriophage displaying BLYS binding polypeptides on their surfaces are also provided. Such phage may be routinely generated using techniques known in the art and are useful, for example, as screening reagents and reagents for detecting BLYS.

#### Production and Modification of BLYS Binding Polypeptides

BLYS binding polypeptides of the invention may be produced by chemical synthesis, semi-synthetic methods, and recombinant DNA methodologies known in the art.

In certain embodiments, BLYS binding polypeptides of the present invention are produced by chemical or semi-synthetic methodologies known in the art (see, Kelley et al. in Genetic Engineering Principles and Methods, Setlow, J.K., ed. (Plenum Press, NY., 1990), vol. 12, pp. 1-19; Stewart et al., Solid-Phase Peptide Synthesis, W. H. Freeman Co., San Francisco, 1989). One advantage of these methodologies is that they allow for the incorporation of non-natural amino acid residues into the sequence of the BLYS binding polypeptide.

In preferred embodiments, BLYS binding polypeptides of the invention are chemically synthesized (see, e.g., Merrifield, J. Am. Chem. Soc., 85: 2149 (1963); Houghten, Proc. Natl. Acad. Sci. USA, 82: 5132 (1985)). For example, polypeptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography (see, e.g., Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Co., N.Y., 1983), pp. 50-60). BLYS binding polypeptides can also be synthesized by use of a peptide synthesizer. The composition of the synthetic polypeptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see

Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Co., N.Y., 1983), pp. 34-49). Furthermore, if desired, BLYS binding polypeptides of the invention may contain non-classical amino acids or chemical amino acid analogs, which can routinely be introduced during chemical synthesis as a substitution or addition into the BLYS binding polypeptides of the invention. Non-classical amino acids include, but are not-limited to, the D-isomers of the common amino acids, 2,4-diaminobutyric acid, alpha-aminoisobutyric acid, 4-aminobutyric acid (4Abu), 2-aminobutyric acid (Abu), 6-aminohexanoic acid (epsilon-Ahx), 2-aminoisobutyric acid (Aib), 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, beta-alanine (bAla), fluoro-amino acids, designer amino acids such as beta-methyl amino acids, Calpha-methyl amino acids, Nalpha-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

Solid phase peptide synthesis begins at the carboxy (C) terminus of the putative polypeptide by coupling a protected amino acid to a suitable resin, which reacts with the carboxyl group of the C-terminal amino acid to form a bond that is readily cleaved later, for example, a halomethyl resin such as chloromethyl resin, bromomethyl resin, hydroxymethyl resin, aminomethyl resin, benzhydrylamine resin, or t-alkyloxycarbonyl-hydrazide resin. After removal of the  $\alpha$ -amino protecting group with, for example, trifluoroacetic acid (TFA) in methylene chloride and neutralization with, for example TEA, the next cycle in the synthesis is ready to proceed. The remaining  $\alpha$ -amino and, if necessary, side-chain-protected amino acids are then coupled sequentially in the desired order by condensation to obtain an intermediate compound connected to the resin. Alternatively, some amino acids may be coupled to one another forming an oligopeptide prior to addition to the growing solid phase polypeptide chain.

The condensation between two amino acids, or an amino acid and a peptide, or a peptide and a peptide can be carried out according to condensation methods known in the art, including but not limited to, the azide method, mixed acid anhydride method, DCC (dicyclohexylcarbodiimide) method, active ester method (p-nitrophenyl ester method, BOP [benzotriazole-1-yl-oxy-tris (dimethylamino) phosphonium hexafluorophosphate] method, N-hydroxysuccinic acid imido ester method), and Woodward reagent K method.

Common to chemical synthesis of peptides is the protection or capping (blocking) of the reactive side chain groups of the various amino acid residues with suitable protecting or capping

groups at that site until the group is ultimately removed after the polypeptide chain has been completely assembled. Also common is the protection or capping of the  $\alpha$ -amino group on an amino acid or a fragment while that entity reacts at the carboxyl group followed by the selective removal of the  $\alpha$ -amino-protecting group to allow subsequent reaction to take place at that location. Accordingly, during synthesis, intermediate compounds are produced which includes each of the amino acid residues located in the desired sequence in the peptide chain with various of these residues having side-chain protecting or capping groups. These protecting or capping groups on amino acid side chains are then removed substantially at the same time so as to produce the desired resultant product following purification.

The typical protective, capping, or blocking groups for  $\alpha$ - and  $\epsilon$ -amino side chain groups found in amino acids are exemplified by benzyloxycarbonyl (Z), isonicotinylloxycarbonyl (iNOC), O-chlorobenzyloxycarbonyl [Z(NO<sub>2</sub>)], p-methoxybenzyloxycarbonyl [Z(OMe)], t-butoxycarbonyl (Boc), t-amylloxycarbonyl (Aoc), isobornylloxycarbonyl, adamantylloxycarbonyl, 2-(4-biphenyl)-2-propyloxycarbonyl (Bpoc), 9-fluorenylmethoxycarbonyl (Fmoc), methylsulfonylloxycarbonyl (Msc), trifluoroacetyl, phthalyl, formyl, 2-nitrophenylsulphenyl (NPS), diphenylphosphinothioyl (Ppt), dimethylphosphinothioyl (Mpt), and the like.

Protective, capping, or blocking groups for the carboxyl group of amino acids include, for example, benzyl ester (OBzl), cyclohexyl ester (Chx), 4-nitrobenzyl ester (ONb), t-butyl ester (Obut), 4-pyridylmethyl ester (OPic), and the like. It is usually also desirable that side chain groups of specific amino acids such as arginine, cysteine, and serine, are protected by a suitable protective group as occasion demands. For example, the guanidino group in arginine may be protected with nitro, p-toluenesulfonyl, benzyloxycarbonyl, adamantylloxycarbonyl, p-methoxybenzenesulfonyl, 4-methoxy-2,6-dimethylbenzenesulfonyl (Mds), 1,3,5-trimethylphenylsulfonyl (Mts), and the like. The thiol group in cysteine may be protected with p-methoxybenzyl, triphenylmethyl, acetylaminoethyl ethylcarbamoyle, 4-methylbenzyl, 2,4,6-trimethylbenzyl (Tmb), etc., and the hydroxyl group in the serine can be protected with benzyl, t-butyl, acetyl, tetrahydropyranyl, etc.

After the desired amino acid sequence has been completed, the intermediate polypeptide is removed from the resin support by treatment with a reagent, such as liquid HF and one or more thio-containing scavengers, which cleaves the peptide molecule from the resin and all the remaining side-chain protecting groups. Following HF cleavage, the protein sequence is washed

with ether, transferred to a large volume of dilute acetic acid, and stirred at pH adjusted to about 8.0 with ammonium hydroxide. Upon pH adjustment, the polypeptide takes its desired conformational arrangement.

By way of example but not by way of limitation, polypeptides of the invention can be chemically synthesized and purified as follows: Peptides can be synthesized by employing the N-alpha-9-fluorenylmethyloxycarbonyl or Fmoc solid phase peptide synthesis chemistry using a Rainin Symphony Multiplex Peptide Synthesizer. The standard cycle used for coupling of an amino acid to the peptide-resin growing chain generally includes: (1) washing the peptide-resin three times for 30 seconds with N,N-dimethylformamide (DMF); (2) removing the Fmoc protective group on the amino terminus by deprotection to with 20% piperidine in DMF by two washes for 15 minutes each, during which process mixing is effected by bubbling nitrogen through the reaction vessel for one second every 10 seconds to prevent peptide-resin settling; (3) washing the peptide-resin three times for 30 seconds with DMF; (4) coupling the amino acid to the peptide resin by addition of equal volumes of a 250 mM solution of the Fmoc derivative of the appropriate amino acid and an activator mix consisting of 400 mM N-methylmorpholine and 250 mM (2-(1H-benzotriazol-1-yl))-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in DMF; (5) allowing the solution to mix for 45 minutes; and (6) washing the peptide-resin three times for 30 seconds of DMF. This cycle can be repeated as necessary with the appropriate amino acids in sequence to produce the desired peptide. Exceptions to this cycle program are amino acid couplings predicted to be difficult by nature of their hydrophobicity or predicted inclusion within a helical formation during synthesis. For these situations, the above cycle can be modified by repeating step 4 a second time immediately upon completion of the first 45 minute coupling step to "double couple" the amino acid of interest. Additionally, in the first coupling step in peptide synthesis, the resin can be allowed to swell for more efficient coupling by increasing the time of mixing in the initial DMF washes to three 15 minute washes rather than three 30 second washes.

After peptide synthesis, the peptide can be cleaved from the resin as follows: (1) washing the peptide-resin three times for 30 seconds with DMF; (2) removing the Fmoc protective group on the amino terminus by washing two times for 15 minutes with 20% piperidine in DMF; (3) washing the peptide-resin three times for 30 seconds with DMF; and (4) mixing a cleavage cocktail consisting of 95% trifluoroacetic acid (TFA), 2.4% water, 2.4% phenol, and 0.2%



triisopropylsilane with the peptide-resin for two hours, then filtering the peptide in the cleavage cocktail away from the resin, and precipitating the peptide out of solution by addition of two volumes of ethyl ether. Specifically, to isolate the peptide, the ether-peptide solution can be allowed to sit at -20° C for 20 minutes, then centrifuged at 6,000 × G for 5 minutes to pellet the peptide, and the peptide can be washed three times with ethyl ether to remove residual cleavage cocktail ingredients. The final peptide product can be purified by reversed phase high pressure liquid chromatography (RP-HPLC) with the primary solvent consisting of 0.1% TFA and the eluting buffer consisting of 80% acetonitrile and 0.1% TFA. The purified peptide can then be lyophilized to a powder.

In other specific embodiments, branched versions of the BLYS binding polypeptides described herein are provided, e.g., by substituting one or more amino acids within the BLYS binding polypeptide sequence with an amino acid or amino acid analog with a free side chain capable of forming a peptide bond with one or more amino acids (and thus capable of forming a “branch”).

Branched peptides may be prepared by any method known in the art for covalently linking any naturally occurring or synthetic amino acid to any naturally occurring or synthetic amino acid in a peptide chain which has a side chain group able to react with the amino or carboxyl group on the amino acids so as to become covalently attached to the peptide chain. In particular, amino acids with a free amino side chain group, such as, but not limited to, diaminobutyric acid, lysine, arginine, ornithine, diaminopropionic acid and citrulline, can be incorporated into a peptide so that an amino acid can form a branch therewith, for example, by forming a peptide bond to the free amino side group, from that residue. Alternatively, amino acids with a free carboxyl side chain group, such as, but not limited to, glutamic acid, aspartic acid and homocitrulline, can be incorporated into the peptide so that an amino acid can form a branch therewith, for example, by forming a peptide bond to the free carboxyl side group, from that residue. The amino acid forming the branch can be linked to a side chain group of an amino acid in the peptide chain by any type of covalent bond, including, but not limited to, peptide bonds, ester bonds and disulfide bonds. In a specific embodiment, amino acids, such as those described above, that are capable of forming a branch point, are substituted for BLYS binding polypeptide residues within a peptide including a BLYS binding polypeptide sequence.

Branched peptides can be prepared by any method known in the art. For example, but

not by way of limitation, branched peptides can be prepared as follows: (1) the amino acid to be branched from the main peptide chain can be purchased as an N-alpha-tert-butyloxycarbonyl (Boc) protected amino acid pentafluorophenyl (Opfp) ester and the residue within the main chain to which this branched amino acid will be attached can be an N-Fmoc-alpha-gamma-  
5 diaminobutyric acid; (2) the coupling of the Boc protected amino acid to diaminobutyric acid can be achieved by adding 5 grams of each precursor to a flask containing 150 ml DMF, along with 2.25 ml pyridine and 50 mg dimethylaminopyridine and allowing the solution to mix for 24 hours; (3) the peptide can then be extracted from the 150 ml coupling reaction by mixing the reaction with 400 ml dichloromethane (DCM) and 200 ml 0.12N HCl in a 1 liter separatory  
10 funnel, and allowing the phases to separate, saving the bottom aqueous layer and re-extracting the top layer two more times with 200 ml 0.12N HCl; (4) the solution containing the peptide can be dehydrated by adding 2-5 grams magnesium sulfate, filtering out the magnesium sulfate, and evaporating the remaining solution to a volume of about 2-5 ml; (5) the dipeptide can then be precipitated by addition of ethyl acetate and then 2 volumes of hexanes and then collected by filtration and washed two times with cold hexanes; and (6) the resulting filtrate can be lyophilized to achieve a light powder form of the desired dipeptide. Branched peptides prepared by this method will have a substitution of diaminobutyric acid at the amino acid position which is branched. Branched peptides containing an amino acid or amino acid analog substitution other than diaminobutyric acid can be prepared analogously to the procedure described above, using the N-Fmoc coupled form of the amino acid or amino acid analog.

In a preferred embodiment, the BLyS binding polypeptide of the invention is a cyclic peptide. Cyclization can be, for example, but not by way of limitation, via a disulfide bond between two cysteine residues or via an amide linkage. For example, but not by way of limitation, disulfide bridge formation can be achieved by (1) dissolving the purified peptide at a  
25 concentration of between 0.1-0.5 mg/ml in 0.01 M ammonium acetate, pH 7.5; (2) adding to the dissolved peptide 0.01 M potassium ferricyanide dropwise until the solution appears pale yellow in color and allowing this solution to mix for 24 hours; (3) concentrating the cyclized peptide to 5-10 ml of solution, repurifying the peptide by reverse phase-high pressure liquid chromatography (RP-HPLC) and finally lyophilizing the peptide. In a specific embodiment, in  
30 which the peptide does not contain two appropriately situated cysteine residues, cysteine residues can be introduced at the amino-terminus and/or carboxy-terminus and/or internally such that the

peptide to be cyclized contains two cysteine residues spaced such that the residues can form a disulfide bridge. Alternatively, a cyclic peptide can be obtained by generating an amide linkage using, for example but not limited to, the following protocol: An allyl protected amino acid, such as aspartate, glutamate, asparagine or glutamine, can be incorporated into the peptide as the first amino acid, and then the remaining amino acids are coupled on. The allyl protective group can be removed by a two hour mixing of the peptide-resin with a solution of tetrakis(triphenylphosphine) palladium (0) in a solution of chloroform containing 5% acetic acid and 2.5% N-methylmorpholine. The peptide resin can be washed three times with 0.5% N,N-diisopropylethylamine (DIEA) and 0.5% sodium diethyldithiocarbamate in DMF. The amino terminal Fmoc group on the peptide chain can be removed by two incubations for 15 minutes each in 20% piperidine in DMF, and washed three times with DMF for 30 seconds each. The activator mix, N-methylmorpholine and HBTU in DMF, can be brought onto the column and allowed to couple the free amino terminal end to the carboxyl group generated by removal of the allyl group to cyclize the peptide. The peptide can be cleaved from the resin as described in the general description of chemical peptide synthesis above and the peptide purified by reverse phase-high pressure liquid chromatography (RP-HPLC). In a specific embodiment, in which the peptide to be cyclized does not contain an allyl protected amino acid, an allyl protected amino acid can be introduced into the sequence of the peptide, at the amino-terminus, carboxy-terminus or internally, such that the peptide can be cyclized.

In addition, according to certain embodiments, it is preferable that the BLYS binding polypeptides of the invention are produced having or retaining an amino terminal (N-terminal) and/or a carboxy terminal (C-terminal) capping group, which may protect the N-terminal or C-terminal amino acid from undesirable chemical reactions during use or which may permit further conjugations or manipulations of the binding polypeptide, for example, in conjugating the binding polypeptide to a chromatographic support resin or matrix or to another peptide to tether the binding polypeptide to a resin or support. Such N-terminal and C-terminal groups may also be used to label or tag the binding polypeptide to detect bound complexes or to locate the binding polypeptide (whether bound or unbound to a BLYS target protein) for example, at some point in a separation procedure. Accordingly, a BLYS binding polypeptide of the invention synthesized in its final form for use in a detection or separation procedure may contain an N-terminal and/or a C-terminal capping group. A particularly preferred N-terminal capping group,

which may be present or retained in binding polypeptides of the invention, is an acetyl group (Ac). A particularly preferred C-terminal capping group, which may be present or retained in binding polypeptides of the invention, is an amide group. In a further preferred embodiment, the BLYS binding polypeptides of the invention have an acetyl group as an N-terminal capping group and an amide group as a C terminal capping group.

The BLYS binding polypeptides of the invention may also be prepared commercially by companies providing polypeptide synthesis as a service (e.g., BACHEM Bioscience, Inc., King of Prussia, PA; Quality Controlled Biochemicals, Inc., Hopkinton, MA).

The nucleic acid sequence encoding a BLYS binding polypeptide of the invention can be produced and isolated using well-known techniques in the art. In one example, nucleic acids encoding the BLYS binding polypeptides of the invention are chemically synthesized based on knowledge of the amino acid sequence of the BLYS binding polypeptide (preferably the sequence is codon optimized to the host system in which the polypeptide will be expressed). In another example, nucleic acids encoding a BLYS binding polypeptide are obtained by screening an expression library (e.g., a phage display library) to identify phage expressing BLYS binding polypeptides, and isolating BLYS binding polypeptide encoding nucleic acid sequences from the identified library member (e.g., via polymerase chain reaction methodology using primers flanking the polypeptide encoding sequences).

The present invention also relates to vectors which include nucleic acid sequences encoding the BLYS binding polypeptides of the invention, host cells which are genetically engineered with the recombinant vectors, or which are otherwise engineered to produce the polypeptides of the invention, and the production of BLYS binding polypeptides, or fragments thereof, by recombinant, chemical or synthetic techniques.

Thus, according to the present invention, BLYS binding polypeptides can also be obtained by recombinant expression techniques. (See, e.g., Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Glover, D. M. (ed.), (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); DNA Cloning: A Practical Approach (MRL Press, Ltd., Oxford, U.K., 1985), Vols. I, II.

To produce a recombinant BLYS binding polypeptide, a nucleic acid sequence encoding the BLYS binding polypeptide is operatively linked to a promoter such that the BLYS binding polypeptide is produced from said sequence. For example, a vector can be introduced into a cell,

within which cell the vector or a portion thereof is expressed, producing the BLYS binding polypeptides. In a preferred embodiment, the nucleic acid is DNA if the source of RNA polymerase is DNA-directed RNA polymerase, but the nucleic acid may also be RNA if the source of polymerase is RNA-directed RNA polymerase or if reverse transcriptase is present in the cell or provided to produce DNA from the RNA. Such a vector can remain episomal or, become chromosomally integrated, as long as it can be transcribed to produce the desired RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be bacteriophage, plasmid, viral, retroviral, or others known in the art, used for replication and expression in bacterial, fungal, plant, insect or mammalian cells. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells. Introduction of the vector construct into the host cell can be effected by techniques known in the art which include, but are not limited to, calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., Basic Methods In Molecular Biology (1986).

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), a-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, for example, stabilization or simplified purification of expressed recombinant product.

In one embodiment, the nucleic acid encoding a BLYS binding polypeptide of the invention is operatively associated with an appropriate heterologous regulatory element (e.g., promoter or enhancer), such as, the phage lambda PL promoter, the *E. coli lac*, *trp*, *phoA*, and *tac*

promoters, the SV40 early and late promoters, and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan.

As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells (e.g., *Saccharomyces cerevisiae* or *Pichia pastoris* (ATCC Accession No. 201178)); insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, 293, NSO and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

The host cell can be a higher eukaryotic cell, such as a mammalian cell (e.g., a human derived cell), or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. The host strain may be chosen which modulates the expression of the inserted nucleic acid sequences encoding the BLYS polypeptides of the invention, or modifies and processes the BLYS binding polypeptide in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus expression of the genetically engineered polypeptide may be controlled. Furthermore, different host cells have characteristics and specific mechanisms for the translational and post-translational processing and modification (e.g., phosphorylation, cleavage) of proteins. Appropriate cell lines can be chosen to ensure the desired modifications and processing of the foreign protein expressed. Selection of appropriate vectors and promoters for expression in a host cell is a well-known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will preferably comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*,

although others may also be employed as a matter of choice. As a representative, but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well-known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed. Among vectors preferred for use in bacteria are pHE4-5 (ATCC Accession No. 209311) and variations thereof, pQE70, pQE60 and pQE-9, available from QIAGEN, Inc.; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Preferred expression vectors for use in yeast systems include, but are not limited to, pYES2, pYD1, pTEF1/Zeo, pYES2/GS, pPICZ, pGAPZ, pGAPZalpha, pPIC9, pPIC3.5, pHIL-D2, pHIL-S1, pPIC3.5K, pPIC9K, and PAO815 (all available from Invitrogen, Carlsbad, CA). Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL (available from Pharmacia). Other suitable vectors will be readily apparent to the skilled artisan.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

In one embodiment, the yeast *Pichia pastoris* is used to express a BLyS binding polypeptide in a eukaryotic system. *Pichia pastoris* is a methylotrophic yeast which can metabolize methanol as its sole carbon source. A main step in the methanol metabolism pathway is the oxidation of methanol to formaldehyde using O<sub>2</sub>. This reaction is catalyzed by the enzyme alcohol oxidase. In order to metabolize methanol as its sole carbon source, *Pichia pastoris* must generate high levels of alcohol oxidase due, in part, to the relatively low affinity of

alcohol oxidase for O<sub>2</sub>. Consequently, in a growth medium depending on methanol as a main carbon source, the promoter region of one of the two alcohol oxidase genes (*AOX1*) is highly active. In the presence of methanol, alcohol oxidase produced from the *AOX1* gene comprises up to approximately 30% of the total soluble protein in *Pichia pastoris*. See, Ellis et al., Mol. Cell. Biol., 5:1111-21 (1985); Koutz et al., Yeast, 5:167-77 (1989); Tschopp et al., Nucl. Acids Res., 15:3859-76 (1987). Thus, a heterologous coding sequence, such as, for example, a polynucleotide encoding a BLYS binding polypeptide of the present invention, under the transcriptional regulation of all or part of the *AOX1* regulatory sequence is expressed at exceptionally high levels in *Pichia* yeast grown in the presence of methanol.

In one example, the plasmid vector pPIC9K is used to express DNA encoding a BLYS binding polypeptide of the invention, as set forth herein, in a *Pichea* yeast system essentially as described in "*Pichia* Protocols: Methods in Molecular Biology," D.R. Higgins and J. Cregg, eds. (The Humana Press, Totowa, N.J., 1998). This expression vector allows expression and secretion of a BLYS binding polypeptide of the invention by virtue of the strong *AOX1* promoter linked to the *Pichia pastoris* alkaline phosphatase (PHO) secretory signal peptide (i.e., leader) located upstream of a multiple cloning site.

Many other yeast vectors may be used in place of pPIC9K, such as, pYES2, pYD1, pTEF1/Zeo, pYES2/GS, pPICZ, pGAPZ, pGAPZalpha, pPIC9, pPIC3.5, pHIL-D2, pHIL-S1, pPIC3.5K, and PAO815, as one skilled in the art would readily appreciate, as long as the proposed expression construct provides appropriately located signals for transcription, translation, secretion (if desired), and the like, including an in-frame AUG as required.

In one embodiment, high-level expression of a heterologous coding sequence, such as, for example, a nucleic acid encoding a BLYS binding polypeptide of the invention, may be achieved by cloning the heterologous nucleic acid sequence of the invention into an expression vector such as, for example, pGAPZ or pGAPZalpha, and growing the yeast culture in the absence of methanol.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp



100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney  
5 fibroblasts, described by Gluzman (Cell, 23:175 (1981)), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, 293, NSO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking  
10 nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The host cells described herein may be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, cell-free translation systems can also be employed to produce the polypeptides of the invention using RNAs derived from the DNA constructs of the present invention.

The polypeptides of the invention may be expressed or synthesized in a modified form, such as a fusion protein (comprising the polypeptide joined via a peptide bond to a heterologous protein sequence (of a different protein)), and may include not only secretion signals, but also additional heterologous functional regions. Such a fusion protein can be made by ligating  
20 polynucleotides of the invention and the desired nucleic acid sequence encoding the desired amino acid sequence to each other, by methods known in the art, in the proper reading frame, and expressing the fusion protein product by methods known in the art. Alternatively, such a fusion protein can be made by protein synthetic techniques, e.g., by use of a peptide synthesizer. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be  
25 added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are  
30 familiar and routine techniques in the art. Particular mention is made of the hexa-histidine polypeptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue,

Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz et al., Proc. Natl. Acad. Sci. USA, 86:821-824 (1989), for instance, hexahistidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the hemagglutinin"HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., Cell, 37:767 (1984)) and the "flag" tag (DYKDDDDK, (SEQ ID NO:183) Stratagene, La Jolla, CA).

In one embodiment, nucleic acids encoding a BLYS binding polypeptides of the invention may be fused to the pelB pectate lyase signal sequence to increase the efficiency to expression and purification of such polypeptides in Gram-negative bacteria. See, U.S. Patent Nos. 5,576,195 and 5,846,818, the contents of which are herein incorporated by reference in their entireties.

Polypeptides of the present invention include products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

The invention encompasses BLYS binding polypeptides which are modified during or after synthesis or translation, e.g., by glycosylation, acetylation, benzoylation, phosphorylation, amidation, pegylation, formylation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, ubiquitination, etc. (See, for instance, Creighton, Proteins: Structures and Molecular Properties, 2d Ed. (W.H. Freeman and Co., N.Y., 1992); Postranslational Covalent Modification of Proteins, Johnson, ed. (Academic Press, New York, 1983), pp. 1-12; Seifter et al., Meth. Enzymol., 182:626-646 (1990); Rattan et al., Ann. NY Acad. Sci., 663:48-62 (1992).) In specific embodiments, the peptides are acetylated at the N-terminus and/or amidated at the C-terminus.

In further embodiments, BLYS binding polypeptides of the invention containing two or more residues that have the potential to interact, such as for example, two cysteine residues in a

polypeptide, may be treated under oxidizing conditions or other conditions that promote interaction of these residues (e.g., disulfide bridge formation).

Further BLYS binding polypeptide modifications encompassed by the invention include, for example, any of numerous chemical modifications carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH<sub>4</sub>, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc.

Additional post-translational /post-synthesis modifications encompassed by the invention include, for example, e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of procaryotic host cell expression.

Also provided by the invention are chemically modified derivatives of BLYS binding polypeptides of the invention which may provide additional advantages such as increased affinity, decreased off-rate, solubility, stability and *in vivo* or *in vitro* circulating time of the polypeptide, or decreased immunogenicity (see, U.S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 1 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any, on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog). For example, the polyethylene glycol may have an average molecular weight of about 200, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10,000, 10,500, 11,000, 11,500, 12,000,

12,500, 13,000, 13,500, 14,000, 14,500, 15,000, 15,500, 16,000, 16,500, 17,000, 17,500, 18,000, 18,500, 19,000, 19,500, 20,000, 25,000, 30,000, 35,000, 40,000, 50,000, 55,000, 60,000, 65,000, 70,000, 75,000, 80,000, 85,000, 90,000, 95,000, or 100,000 kDa.

As noted above, the polyethylene glycol may have a branched structure. Branched polyethylene glycols are described, for example, in U.S. Patent No. 5,643,575; Morpurgo et al., Appl. Biochem. Biotechnol., 56:59-72 (1996); Vorobjev et al., Nucleosides Nucleotides, 18:2745-2750 (1999); and Caliceti et al., Bioconjug. Chem., 10:638-646 (1999), the disclosures of each of which are incorporated herein by reference.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the BLYS binding polypeptide with consideration of effects on functional domains of the polypeptide. There are a number of attachment methods available to those skilled in the art, e.g., EP 0 401 384, herein incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol., 20:1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include, for example, lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecules. In a preferred embodiment, the polyethylene glycol molecule is attached at an amino group, such as attachment at the N-terminus or to a lysine side chain amino group.

As suggested above, polyethylene glycol may be attached to polypeptides via linkage to any of a number of amino acid residues. For example, polyethylene glycol can be linked to a polypeptide via covalent bonds to lysine, histidine, aspartic acid, glutamic acid, or cysteine residues. One or more reaction chemistries may be employed to attach polyethylene glycol to specific amino acid residues (e.g., lysine, histidine, aspartic acid, glutamic acid, or cysteine) of the polypeptide or to more than one type of amino acid residue (e.g., lysine, histidine, aspartic acid, glutamic acid, cysteine and combinations thereof) of the polypeptide.

One may specifically desire proteins chemically modified at the N-terminus. Using polyethylene glycol as an illustration, one may select from a variety of polyethylene glycol

molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to polypeptide molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated polypeptide. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated polypeptide molecules. Selective N-terminal modification of proteins may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminus) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved.

As indicated above, pegylation of the polypeptides of the invention may be accomplished by any number of means. For example, polyethylene glycol may be attached to the protein either directly or by an intervening linker. Linkerless systems for attaching polyethylene glycol to proteins are described in Delgado et al., Crit. Rev. Thera. Drug Carrier Sys., 9:249-304 (1992); Francis et al., Intern. J. of Hematol., 68:1-18 (1998); U.S. Patent No. 4,002,531; U.S. Patent No. 5,349,052; WO 95/06058; and WO 98/32466, the disclosures of each of which are incorporated herein by reference.

One system for attaching polyethylene glycol directly to amino acid residues of polypeptides without an intervening linker employs tresylated MPEG, which is produced by the modification of monomethoxy polyethylene glycol (MPEG) using tresylchloride ( $\text{ClSO}_2\text{CH}_2\text{CF}_3$ ). Upon reaction of protein with tresylated MPEG, polyethylene glycol is directly attached to amine groups of the polypeptide. Thus, the invention includes polypeptide-polyethylene glycol conjugates produced by reacting polypeptides of the invention with a polyethylene glycol molecule having a 2,2,2-trifluoroethane sulphonyl group.

Polyethylene glycol can also be attached to polypeptides using a number of different intervening linkers. For example, U.S. Patent No. 5,612,460, the entire disclosure of which is incorporated herein by reference, discloses urethane linkers for connecting polyethylene glycol to polypeptides. Polypeptide-polyethylene glycol conjugates wherein the polyethylene glycol is attached to the polypeptide by a linker can also be produced by reaction of polypeptides with compounds such as MPEG-succinimidylsuccinate, MPEG activated with

1,1'-carbonyldiimidazole, MPEG-2,4,5-trichlorophenylcarbonate, MPEG-p-nitrophenolcarbonate, and various MPEG-succinate derivatives. A number of additional polyethylene glycol derivatives and reaction chemistries for attaching polyethylene glycol to polypeptides are described in WO 98/32466, the entire disclosure of which is incorporated herein by reference. Pegylated BLYS binding polypeptide products produced using the reaction chemistries set out herein are included within the scope of the invention.

The number of polyethylene glycol moieties attached to each polypeptide of the invention (i.e., the degree of substitution) may also vary. For example, the pegylated polypeptides of the invention may be linked, on average, to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 17, 20, or more polyethylene glycol molecules. Similarly, the average degree of substitution may range within ranges such as 1-3, 2-4, 3-5, 4-6, 5-7, 6-8, 7-9, 8-10, 9-11, 10-12, 11-13, 12-14, 13-15, 14-16, 15-17, 16-18, 17-19, or 18-20 polyethylene glycol moieties per polypeptide molecule. Methods for determining the degree of substitution are discussed, for example, in Delgado et al., Crit. Rev. Thera. Drug Carrier Sys., 9:249-304 (1992).

#### BLYS Binding Polypeptide Multimers, Conjugates and Fusions

The present invention encompasses multivalent BLYS binding polypeptides. BLYS binding polypeptides may be monomeric, dimeric, trimeric, or higher-order multimers. In a preferred embodiment multivalent BLYS binding polypeptides are homotrimeric. In another preferred embodiment a homotrimeric BLYS binding polypeptide binds a single homotrimeric BLYS.

In another preferred embodiment, monomeric or multimeric BLYS binding polypeptides are conjugated with another polypeptide or other chemical compound. For example, BLYS binding polypeptide(s) may be conjugated to a radioactive or other toxic compound so as to target and destroy cells expressing BLYS.

The present invention also encompasses heteromeric multimers comprised of one or more BLYS binding polypeptides and one or more non-BLYS binding polypeptides or other chemical moieties. Such heteromeric multimers may be monomeric, dimeric, trimeric, tetrameric, pentameric, or higher-order multimers. Heteromeric BLYS binding multimers may be used to target, bind, inhibit, and/or activate responses in cells expressing BLYS and receptors for the heterologous, non-BLYS binding polypeptide or other chemical moiety. Such activated responses may include, for example, apoptosis or other biologically and chemically mediated

forms of cell destruction. Heteromeric BLyS binding multimers may also be used to target BLyS expressing cells so as to introduce a desired molecule or compound to the cells. For example, a heteromeric BLyS binding multimer may be conjugated with a radioactive or otherwise toxic compound so as to kill BLyS expressing cells. As an alternative example, a heteromeric BLyS binding and Adenovirus-binding multimer could be used to specifically target and introduce adenovirus-mediated gene therapeutics into BLyS expressing cells.

BLyS binding polypeptide multimers may be fused or conjugated as homopolymers and heteropolymers using methods known in the art. In a preferred embodiment BLyS binding polypeptides are linked as homomultimers wherein the linker or linkers provide sufficient length and flexibility such that each BLyS binding polypeptide may simultaneously bind an individual BLyS molecule. In another preferred embodiment BLyS binding polypeptides are linked as heteromultimers wherein the linker or linkers provide sufficient length and flexibility such that each BLyS binding polypeptide may simultaneously bind individual BLyS molecules and the heterologous polypeptide or chemical moiety may simultaneously bind to its target. Numerous examples of suitable linker molecules are known in the art. (See, for example, Todorovska et al., J. Immunol. Methods, 248(1-2):47-66 (2001); Mehvar, J. Control Release, 69(1):1-25 (2000); Francis et. al., Int. J. Hematol., 68(1):1-18 (1998).) In specific embodiments, the linker is a member selected from the group consisting of: (a) a peptide linker; (b) a glutamate linker; and (c) a polyethylene glycol linker. The length of linkers to be used according to the methods of the invention may routinely be determined using techniques known in the art. In specific embodiments, the linker is 5-60 angstroms in length. In other embodiments, the linker is 10-50, 10-40, 10-30, or 10-20 angstroms in length. In further embodiments, the linker is about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 angstroms in length. In this context "about" includes the recited length, and/or lengths that are larger or smaller by several (5, 4, 3, 2, or 1) angstroms. In other embodiments, the linker is at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 angstroms in length.

In a preferred embodiment, BLyS binding polypeptides may be fused with human serum albumin (HA). See, e.g., U.S. application Serial No. 09/833,245, filed April 12, 2001, which is hereby incorporated by reference herein. In one embodiment, the albumin fusion protein comprises HA as the N-terminal portion, and a BLyS binding polypeptide as the C-terminal

portion. In another embodiment the albumin fusion protein comprise HA as the C-terminal portion, and a BLyS binding polypeptide as the N-terminal portion.

In other embodiments, the albumin fusion protein has a BLyS binding polypeptide fused to both the N-terminus and the C-terminus of albumin. In one preferred embodiment, the BLyS binding polypeptides fused at the N- and C- termini are the same BLyS binding polypeptides. In another preferred embodiment, the BLyS binding polypeptides fused at the N- and C-termini are different BLyS binding polypeptides. In another preferred embodiment, a BLyS binding polypeptide is fused at either the N- or C- terminus of HA and a different (non-BLyS binding) polypeptide is fused at either the C- or N- terminus, respectively.

In addition to albumin fusion proteins in which the BLyS binding polypeptide(s) is (are) fused to the N-terminus and/or C-terminus of HA, BLyS binding polypeptide/albumin fusion proteins of the invention may also be produced by inserting the BLyS binding polypeptide into an internal region or regions of HA. For instance, within the protein sequence of the HA molecule a number of loops or turns exist between the end and beginning of  $\alpha$ -helices, which are stabilized by disulphide bonds (see Figures 9-11 in U.S. application Serial No. 09/833,245). The loops, as determined from the crystal structure of HA (Fig. 13 of U.S. application Serial No. 09/833,245) (PDB identifiers 1AO6, 1BJ5, 1BKE, 1BM0, 1E7E to 1E7I and 1UOR) for the most part extend away from the body of the molecule. These loops are useful for the insertion, or internal fusion, of therapeutically active peptides (particularly those requiring a secondary structure to be functional) or therapeutic proteins, to essentially generate an albumin molecule with specific biological activity.

Loops in human albumin structure into which binding polypeptides of the invention may be inserted to generate albumin fusion proteins of the invention include: Val54-Asn61, Thr76-Asp89, Ala92-Glu100, Gln170-Ala176, His 247 - Glu252, Glu 266 - Glu277, Glu 280-His288, Ala362-Glu368, Lys439-Pro447, Val462-Lys475, Thr478-Pro486, and Lys560-Thr566. In more preferred embodiments, polypeptides of the invention are inserted into the Val54-Asn61, Gln170-Ala176, and/or Lys560-Thr566 loops of mature human serum albumin (SEQ ID NO:445).

In specific embodiments, BLyS binding polypeptides of the invention are attached to macrocyclic chelators useful for conjugating radiometal ions, including but not limited to,  $^{111}\text{In}$ ,  $^{177}\text{Lu}$ ,  $^{90}\text{Y}$ ,  $^{166}\text{Ho}$ , and  $^{153}\text{Sm}$ , to polypeptides. In a preferred embodiment, the radiometal ion



associated with the macrocyclic chelators attached to BLYS binding polypeptides of the invention is  $^{111}\text{In}$ . In another preferred embodiment, the radiometal ion associated with the macrocyclic chelator attached to BLYS binding polypeptides of the invention is  $^{90}\text{Y}$ . In specific embodiments, the macrocyclic chelator is 1,4,7,10-tetraazacyclododecane- $\text{N,N',N'',N'''}-$  tetraacetic acid (DOTA). In other specific embodiments, the DOTA is attached to the BLYS binding polypeptides of the invention via a linker molecule. Examples of linker molecules useful for conjugating DOTA to a polypeptide are commonly known in the art - see, for example, DeNardo et al., Clin. Cancer Res., 4(10):2483-90 (1998); Peterson et al., Bioconjug. Chem., 10(4):553-7 (1999); and Zimmerman et al, Nucl. Med. Biol., 26(8):943-50 (1999), which are hereby incorporated by reference in their entirety. In addition, U.S. Patents 5,652,361 and 5,756,065, which disclose chelating agents that may be conjugated to antibodies, and methods for making and using them, are hereby incorporated by reference in their entireties. Though U.S. Patents 5,652,361 and 5,756,065 focus on conjugating chelating agents to antibodies, one skilled in the art would be readily able to adapt the method disclosed therein in order to conjugate chelating agents to other polypeptides.

The BLYS binding polypeptides of the invention can be recovered and purified by known methods which include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

The BLYS binding polypeptides may also be modified with a detectable label, including, but not limited to, an enzyme, prosthetic group, fluorescent material, luminescent material, bioluminescent material, radioactive material, positron emitting metal, nonradioactive paramagnetic metal ion, and affinity label for detection and isolation of BLYS target. The detectable substance may be coupled or conjugated either directly to the polypeptides of the invention or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, glucose oxidase or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include biotin, umbelliferone, fluorescein, fluorescein

isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include a radioactive metal ion, e.g., alpha-emitters such as, for example,  $^{213}\text{Bi}$ , or other radioisotopes such as, for example, iodine ( $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^{123}\text{I}$ ,  $^{121}\text{I}$ ), carbon ( $^{14}\text{C}$ ), sulfur ( $^{35}\text{S}$ ), tritium ( $^3\text{H}$ ), indium ( $^{115\text{m}}\text{In}$ ,  $^{113\text{m}}\text{In}$ ,  $^{112}\text{In}$ ,  $^{111}\text{In}$ ), and technetium ( $^{99}\text{Tc}$ ,  $^{99\text{m}}\text{Tc}$ ), thallium ( $^{201}\text{Tl}$ ), gallium ( $^{68}\text{Ga}$ ,  $^{67}\text{Ga}$ ), palladium ( $^{103}\text{Pd}$ ), molybdenum ( $^{99}\text{Mo}$ ), xenon ( $^{133}\text{Xe}$ ), fluorine ( $^{18}\text{F}$ ),  $^{153}\text{Sm}$ ,  $^{177}\text{Lu}$ ,  $^{159}\text{Gd}$ ,  $^{149}\text{Pm}$ ,  $^{140}\text{La}$ ,  $^{175}\text{Yb}$ ,  $^{166}\text{Ho}$ ,  $^{90}\text{Y}$ ,  $^{47}\text{Sc}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{142}\text{Pr}$ ,  $^{105}\text{Rh}$ ,  $^{97}\text{Ru}$ ,  $^{68}\text{Ge}$ ,  $^{57}\text{Co}$ ,  $^{65}\text{Zn}$ ,  $^{85}\text{Sr}$ ,  $^{32}\text{P}$ ,  $^{153}\text{Gd}$ ,  $^{169}\text{Yb}$ ,  $^{51}\text{Cr}$ ,  $^{54}\text{Mn}$ ,  $^{75}\text{Se}$ ,  $^{113}\text{Sn}$ , and  $^{117}\text{Sn}$ .

In a specific embodiment, BLYS binding polypeptides of the invention are labeled with biotin.

#### Uses of the Binding Polypeptides and Recombinant Bacteriophage of the Invention

The BLYS binding polypeptides described herein are especially useful to detect, isolate, or remove BLYS target proteins in solutions. Such solutions may be simple dispersions or solutions of BLYS and/or BLYS-like polypeptide in water or aqueous buffer or more complex solutions, such as, a blood and other biological fluids, tissue homogenates cell extracts, or biopsy samples, and cell culture media containing BLYS or BLYS-like polypeptides. Biological fluids include, but are not limited to sera, plasma, lymph, blood, blood fractions urine, synovial fluid, spinal fluid, saliva, and mucous.

In one embodiment, the present invention provides a method for detecting a BLYS protein and/or a BLYS-like polypeptide in a solution comprising contacting the solution with a BLYS binding polypeptide of the invention and detecting binding of BLYS or BLYS-like polypeptide to the BLYS binding polypeptide. The BLYS binding polypeptide may be either free or immobilized. Preferably, the BLYS binding polypeptide is a polypeptide immobilized on a solid surface or chromatographic material or the well of a plastic microtiter assay dish.

Another embodiment of the present invention is a method for isolating BLYS protein and/or BLYS-like polypeptide from a solution containing it, comprising:

- (a) contacting the solution with a BLYS binding polypeptide under conditions that permit binding of BLYS and/or BLYS-like polypeptides to BLYS binding polypeptide, and
- (b) recovering the BLYS and/or BLYS-like polypeptides.

A further embodiment of the present invention is a method for isolating BLyS protein and/or BLyS-like polypeptide from a solution containing it, comprising:

- (a) contacting the solution with a BLyS binding polypeptide under conditions that permit binding of BLyS and/or BLyS-like polypeptides to BLyS binding polypeptide, and
- (b) separating the complex(es) formed by the BLyS binding polypeptide and BLyS and/or BLyS-like polypeptides from other components of the solution.

Preferably such method also includes the further steps of:

- (c) dissociating the BLyS binding polypeptide from the BLyS and/or BLyS-like polypeptides, and
- (d) recovering the dissociated, BLyS and/or BLyS-like polypeptide.

The invention also provides for kits containing a binding polypeptide of the invention for use in methods of detecting or isolating BLyS and/or BLyS-like polypeptides.

According to the invention, detection or isolation of BLyS target proteins comprises contacting a solution containing a BLyS target protein with a BLyS binding polypeptide. Depending on the particular application, the BLyS binding polypeptide may be free in solution or immobilized on a solid support or chromatographic material. Sufficient time is allowed to permit binding between the BLyS target protein and the binding polypeptides, and non-binding components in the solution or mixture are removed or washed away. The formation of a binding complex between the binding polypeptide and the BLyS target protein can then be detected, for example, by detecting the signal from a label on the binding polypeptide, which is one component of the binding complex. A label may be any label that generates a signal that can be detected by standard methods, such as a fluorescent label, a radioactive compound, or an enzyme that reacts with a substrate to generate a detectable signal. Suitable such labels are discussed above. A phage binding polypeptide according to the invention, that is, a recombinant phage displaying a BLyS binding polypeptide on its surface, may form a complex with BLyS and/or BLyS-like polypeptides that is detectable as a precipitate or sediment in a reaction tube, which can be detected visually after settling or centrifugation. Alternatively, a sandwich-type assay may be used, wherein a BLyS binding polypeptide is immobilized on a solid support such as a plastic tube or well, or a chromatographic support matrix such as agarose beads, then the solution suspected of containing the BLyS target is contacted with the immobilized binding polypeptide and non-binding materials or components are removed or washed away.

The binding polypeptides according to this invention are particularly useful for detection and/or isolation of BLyS and/or BLyS-like polypeptides by affinity chromatography methods. Any conventional method of chromatography may be employed. Preferably, a BLyS binding polypeptide of the invention will be immobilized on a solid support suitable, for example, for packing a chromatography column. The immobilized BLyS binding polypeptide affinity ligand can then be loaded or contacted with a feed stream under conditions favorable to formation of binding polypeptide/BLyS (or BLyS-like polypeptide) complexes. Non-binding materials can be washed away. Examples of suitable wash conditions can readily be determined by one of skill in the art and include but are not limited to [PBS/0.01% Tween 20, pH7.2] and [1M NaCl/10mM Tris, pH7.5]. Tris wash buffers may be preferable since phosphates can precipitate in 50% ethylene glycol. In general, non-limiting terms, wash buffers are pH7.0, optionally containing 0.0 to 1.5 M NaCl, more preferably 1M NaCl. Additionally, wash buffers may optionally contain a mild detergent, such as, for example, Tween 20, Tween 80, or NP-80. BLyS or BLyS-like polypeptide can be eluted from the BLyS binding polypeptide by introducing solution conditions that favor dissociation of the binding complex. Suitable elution solutions can readily be determined by one of skill in the art and include but are not limited to [50% ethylene glycol/100mM NaOAc]. By way of non-limiting example, useful elution buffers, for the purposes of the present invention contain 40-60% ethylene glycol, preferably 50% ethylene glycol.; and 50-100mM NaOAc with a pH in the range of pH 4 – pH7 , more preferably, pH 4 – pH 6 and most preferably pH 4.5 – pH 5.5. Preferably, a fast flow affinity chromatographic technique is used to bind the molecules and from which purified BLyS or BLyS-like polypeptides are eluted.

Alternatively, batch chromatography can be carried out by mixing a solution containing the BLyS target and the BLyS binding polypeptide, then isolating complexes of the BLyS target and the binding polypeptides. For this type of separation, many methods are known. For example, the binding polypeptide may be immobilized on a solid support such as beads, then separated from the feed stream along with the BLyS target by filtration. In another example, the BLyS binding polypeptide may be modified with its own affinity tag, such as a polyHis tail or streptavidin binding region, which can be used to isolate the binding polypeptide after complexes have formed using an immobilized metal affinity chromatographic resin or streptavidin-coated

substrate. Once separated, the BLyS target can be released from the binding polypeptide under elution conditions and recovered in a purified form.

Methods of producing BLyS or a BLyS-like polypeptides usually yield BLyS or BLyS-like polypeptides in a feed stream that additionally contains impurities (with respect to the BLyS target). One purpose of the present invention is to produce BLyS binding polypeptides and preparations (such as affinity chromatography media or surfaces) comprising BLyS binding polypeptides that allow rapid and highly specific purification of BLyS target proteins from a feed stream. BLyS binding polypeptides obtained herein may easily be tailored to isolate BLyS target protein from a particular feed stream, using or routinely modifying conditions and techniques known in the art. If an alternate production method for BLyS is used, producing a different feed stream, a different set of BLyS binding polypeptides and/or conditions may be necessary to achieve the same level of purification. The new set of BLyS binding polypeptides and/or conditions can be readily obtained following or modifying procedures outlined herein, or otherwise known in the art.

### Kits

The present invention is also directed to an assay kit which can be useful in screening for the presence of BLyS and/or quantitating BLyS concentrations in a fluid, such as, for example, a biological fluid (e.g., blood, serum, or synovial fluid).

In a particular embodiment of the present invention, an assay kit is contemplated which comprises in one or more containers of BLyS binding polypeptide(s) according to the invention and, optionally, a detection means for determining the presence of a BLyS target/BLyS binding polypeptide interaction or the absence thereof. The kit further optionally contains BLyS protein that may be used, for example as a control or standard. The BLyS binding polypeptide may be free or expressed on the surface of a host cell or on the surface of a bacteriophage.

In a specific embodiment, either the BLyS binding polypeptide or the BLyS protein is labeled. As further discussed herein, a wide range of labels can be used in accordance with the present invention, including but not limited to conjugating the recognition unit to biotin by conventional means. Alternatively, the label may comprise, e.g., a fluorogen, an enzyme, an epitope, a chromogen, or a radionuclide. Preferably, the biotin is conjugated by covalent attachment to either the BLyS binding polypeptide or the BLyS protein. Preferably, the BLyS

binding polypeptide is immobilized on a solid support. The detection means employed to detect the label will depend on the nature of the label and can be any known in the art, e.g., film to detect a radionuclide; an enzyme substrate that gives rise to a detectable signal to detect the presence of an enzyme; antibody to detect the presence of an epitope, etc.

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#### Methods of Screening for BLyS Binding Molecules

The present invention also encompasses screening methods for identifying polypeptides and nonpolypeptides that bind BLyS, and the BLyS binding molecules identified thereby.

This method comprises the steps of:

- 10 (a) contacting a BLyS protein or BLyS-like protein with a plurality of molecules; and  
(b) identifying a molecule that binds the BLyS protein or BLyS-like protein.

The step of contacting the BLyS protein or BLyS-like protein with the plurality of molecules may be effected in a number of ways. For example, one may contemplate immobilizing the BLyS protein or BLyS-like protein on a solid support and bringing a solution of the plurality of molecules in contact with the immobilized BLyS protein or BLyS-like protein. Such a procedure would be akin to an affinity chromatographic process, with the affinity matrix being comprised of the immobilized BLyS protein or BLyS-like polypeptide. The molecules having a selective affinity for the BLyS protein or BLyS-like polypeptide can then be purified by affinity selection. The nature of the solid support, process for attachment of the BLyS protein or BLyS-like polypeptide to the solid support, solvent, and conditions of the affinity isolation or selection are largely conventional and well known to those of ordinary skill in the art.

Alternatively, one may also separate a plurality of polypeptides into substantially separate fractions comprising a subset of or individual polypeptides. For instance, one can separate the plurality of polypeptides by gel electrophoresis, column chromatography, or like method known to those of ordinary skill for the separation of polypeptides. The individual polypeptides can also be produced by a transformed host cell in such a way as to be expressed on or about its outer surface (e.g., a recombinant phage). Individual isolates can then be "probed" by the BLyS protein or BLyS-like polypeptide, optionally in the presence of an inducer should one be required for expression, to determine if any selective affinity interaction takes place between the BLyS protein or BLyS-like protein and the individual clone. Prior to contacting the BLyS protein or BLyS-like protein with each fraction comprising individual polypeptides, the

polypeptides could first be transferred to a solid support for additional convenience. Such a solid support may simply be a piece of filter membrane, such as one made of nitrocellulose or nylon. In this manner, positive clones could be identified from a collection of transformed host cells of an expression library, which harbor a DNA construct encoding a polypeptide having a selective affinity for BLYS protein or BLYS-like protein. Furthermore, the amino acid sequence of the polypeptide having a selective affinity for the BLYS protein or BLYS-like protein can be determined directly by conventional means or the coding sequence of the DNA encoding the polypeptide can frequently be determined more conveniently. The primary sequence can then be deduced from the corresponding DNA sequence. If the amino acid sequence is to be determined from the polypeptide itself, one may use microsequencing techniques. The sequencing technique may include mass spectroscopy.

In certain situations, it may be desirable to wash away any unbound BLYS protein or BLYS-like protein, or alternatively, unbound polypeptides, from a mixture of the BLYS protein or BLYS-like protein and the plurality of polypeptides prior to attempting to determine or to detect the presence of a selective affinity interaction. Such a wash step may be particularly desirable when the BLYS protein or BLYS-like protein or the plurality of polypeptides is bound to a solid support.

The plurality of molecules provided according to this method may be provided by way of diversity libraries, such as random or combinatorial peptide or nonpeptide libraries which can be screened for molecules that specifically bind to BLYS. Many libraries are known in the art that can be used, e.g., chemically synthesized libraries, recombinant (e.g., phage display libraries), and *in vitro* translation-based libraries. Examples of chemically synthesized libraries are described in Fodor et al., Science, 251:767-773 (1991); Houghten et al., Nature, 354:84-86 (1991); Lam et al., Nature, 354:82-84 (1991); Medynski, Bio/Technology, 12:709-710 (1994); Gallop et al., J. Medicinal Chemistry, 37(9):1233-1251 (1994); Ohlmeyer et al., Proc. Natl. Acad. Sci. USA, 90:10922-10926 (1993); Erb et al., Proc. Natl. Acad. Sci. USA, 91:11422-11426 (1994); Houghten et al., Biotechniques, 13:412 (1992); Jayawickreme et al., Proc. Natl. Acad. Sci. USA, 91:1614-1618 (1994); Salmon et al., Proc. Natl. Acad. Sci. USA, 90:11708-11712 (1993); PCT Publication No. WO 93/20242; and Brenner and Lerner, Proc. Natl. Acad. Sci. USA, 89:5381-5383 (1992).

Examples of phage display libraries are described in Scott and Smith, Science, 249:386-390 (1990); Devlin et al., Science, 249:404-406 (1990); Christian et al., J. Mol. Biol., 227:711-718 (1992); Lenstra, J. Immunol. Meth., 152:149-157 (1992); Kay et al., Gene, 128:59-65 (1993); and PCT Publication No. WO 94/18318 dated Aug. 18, 1994.

5 *In vitro* translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated Apr. 18, 1991; and Mattheakis et al., Proc. Natl. Acad. Sci. USA, 91:9022-9026 (1994).

By way of examples of nonpeptide libraries, a benzodiazepine library (see, e.g., Bunin et al., Proc. Natl. Acad. Sci. USA, 91:4708-4712 (1994)) can be adapted for use. Peptoid libraries (see, Simon et al., Proc. Natl. Acad. Sci. USA, 89:9367-9371 (1992)) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (Proc. Natl. Acad. Sci. USA, 91:11138-11142 (1994)).

The variety of non-peptide libraries that are useful in the present invention is great. For example, Ecker and Crooke, Bio/Technology, 13:351-360 (1995) list benzodiazepines, hydantoins, piperazinediones, biphenyls, sugar analogs, beta-mercaptoketones, arylacetic acids, acylpiperidines, benzopyrans, cubanes, xanthines, aminimides, and oxazolones as among the chemical species that form the basis of various libraries.

Non-peptide libraries can be classified broadly into two types: decorated monomers and oligomers. Decorated monomer libraries employ a relatively simple scaffold structure upon which a variety functional groups is added. Often the scaffold will be a molecule with a known useful pharmacological activity. For example, the scaffold might be the benzodiazepine structure.

Non-peptide oligomer libraries utilize a large number of monomers that are assembled together in ways that create new molecular shapes that depend on the order of the monomers. Among the monomer units that have been used are carbamates, pyrrolinones, and morpholinos. Peptoids, peptide-like oligomers in which the side chain is attached to the alpha amino group rather than the alpha carbon, form the basis of another version of non-peptide oligomer libraries. The first non-peptide oligomer libraries utilized a single type of monomer and thus contained a repeating backbone. Recent libraries have utilized more than one monomer, giving the libraries added flexibility.



Screening the libraries can be accomplished by any of a variety of commonly known methods. See, e.g., the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol., 251:215-218; Scott and Smith, 1990, Science, 249:386-390; Fowlkes et al., 1992, BioTechniques, 13:422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA, 89:5393-5397; Yu et al., 1994, Cell, 76:933-945; Staudt et al., 1988, Science, 241:577-580; Bock et al., 1992, Nature, 355:564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA, 89:6988-6992; Ellington et al., 1992, Nature, 355:850-852; U.S. Pat. No. 5,096,815, U.S. Pat. No. 5,223,409, and U.S. Pat. No. 5,198,346, all to Ladner et al.; Rebar and Pabo, 1993, Science, 263:671-673; and CT Publication No. WO 94/18318.

In a specific embodiment, screening to identify a molecule that binds BLYS can be carried out by contacting the library members with a BLYS protein or BLYS-like protein immobilized on a solid phase and harvesting those library members that bind to the BLYS protein or BLYS-like protein. Examples of such screening methods, termed "panning" techniques are described by way of example in Parmley and Smith, 1988, Gene, 73:305-318; Fowlkes et al., 1992, BioTechniques, 13:422-427; PCT Publication No. WO 94/18318; and in references cited herein.

In another embodiment, the two-hybrid system for selecting interacting proteins in yeast (Fields and Song, 1989, Nature, 340:245-246; Chien et al., 1991, Proc. Natl. Acad. Sci. USA, 88:9578-9582) can be used to identify molecules that specifically bind to BLYS or BLYS-like proteins.

An alternative screening method for obtaining new binding moieties capable of binding to BLYS target proteins is to employ a competition assay, in which a BLYS target is bound to a BLYS binding polypeptide according to the present invention, preferably labeled, and then the complex is exposed to one or more test moieties. Successful new BLYS binding moieties will be test moieties capable of effectively competing for binding to the BLYS target in the presence of a known BLYS binder disclosed herein.

Polypeptides specifically binding BLYS target proteins can be conveniently selected from any peptide library, including random peptide libraries, combinatorial peptide libraries, or biased peptide libraries. The term "biased" is used herein to mean that the method of generating the library is manipulated so as to restrict one or more parameters that govern the diversity of the resulting collection of molecules, in this case peptides.

Thus, a truly random peptide library would generate a collection of peptides in which the probability of finding a particular amino acid at a given position of the peptide is the same for all 20 amino acids. A bias can be introduced into the library, however, by specifying, for example, that a lysine occur every fifth amino acid or that positions 4, 8, and 9 of a decapeptide library be fixed to include only arginine. For libraries designed to display a stable loop structure, a peptide sequence may be designed to include two invariant cysteine residues, with all other amino acid positions permitting one or more amino acid residues but excluding cysteine residues. (See, Example 1, *infra*.) Clearly, many types of biases can be contemplated, and the present invention is not restricted to any particular bias. Furthermore, the present invention contemplates specific types of peptide libraries, such as phage displayed peptide libraries and those that utilize a DNA construct comprising a lambda phage vector with a DNA insert.

As mentioned above, in the case of a BLYS binding molecule that is a polypeptide, the polypeptide may have about 6 to less than about 60 amino acid residues, preferably about 6 to about 10 amino acid residues, and most preferably, about 6 to about 22 amino acids. In another embodiment, a BLYS binding polypeptide has in the range of 15-100 amino acids, or 20-50 amino acids.

The selected BLYS binding polypeptide can be produced by chemical synthesis or recombinant expression, as described above.

The specific BLYS binding polypeptides disclosed herein were isolated using phage display technology, to identify BLYS binding polypeptides exhibiting particular preselected binding properties. These BLYS binding polypeptides were isolated initially by screening nine phage display libraries, that is, populations of recombinant bacteriophage transformed to express an exogenous recombinant polypeptide on their surface. In order to isolate new polypeptide binding moieties for a particular target, such as BLYS, screening of peptide libraries, for example using phage display techniques, is especially advantageous, in that very large numbers (e.g.,  $5 \times 10^9$ ) of potential binders can be tested and successful binders isolated in a short period of time.

In order to prepare a phage library of potential binding polypeptides to screen for members of the library that are BLYS binding polypeptides, a candidate binding domain is selected to serve as a structural template for the polypeptides to be displayed in the library. The phage library is made up of polypeptide analogues of this template or "parental binding domain." The parental binding domain is a polypeptide molecule that may be a naturally occurring or

synthetic protein or polypeptide, or polypeptide region or domain of a protein. The parental binding domain may be selected based on knowledge of a known interaction between the parental binding domain and a target protein, but this is not critical. In fact, it is not essential that the parental binding domain have any affinity for a target at all because its purpose is to provide a structure from which a multiplicity of polypeptide analogues (a "library") can be generated, which multiplicity of polypeptide analogues will include one or more binding polypeptides that exhibit the desired binding and release properties with respect to BLYS target proteins (and any other properties selected).

Knowledge of the exact polypeptide that will serve as the parental binding domain, or knowledge of a class of proteins or domains to which the parental binding domain belongs can be useful in determining the conditions under which BLYS binding polypeptides optimally bind BLYS target proteins as well as the conditions under which BLYS binding polypeptides optimally release BLYS target proteins. Similarly, the binding and/or release conditions may be selected with regard to known interactions between a binding domain and the BLYS target protein, for example, to favor the interaction under the binding and/or release conditions, or they may be selected without regard to such known interactions. Likewise, the parental binding domain can be selected taking into account a desired binding and/or release condition or not. It is understood that if the binding domain analogues of a library are unstable under a proposed or desired binding or release condition, no useful binding polypeptides may be obtained.

In selecting the parental binding domain, the most important consideration is how the analogue domains will be presented to the BLYS target protein, that is, in what conformations the BLYS target and the polypeptide analogues will contact one another. In preferred embodiments, for example, the polypeptide analogues will be generated by insertion of synthetic DNA encoding the polypeptide analogue into a replicable genetic package, resulting in display of the domain on the surface of a microorganism, such as M13 phage, using techniques as described in Kay et al., Phage Display of Peptides and Proteins: A Laboratory Manual (Academic Press, Inc.; San Diego 1996) and U.S. Patent No. 5,223,409 (Ladner et al.), incorporated herein by reference. For formation of phage display libraries, it is preferred to use structured polypeptides as the parental binding domain or template, as opposed to unstructured, linear peptides. Mutation of surface residues in a protein domain or polypeptide molecule will usually have little effect on the overall structure or general properties (such as size, stability, and temperature of denaturation) of

the protein; while at the same time mutation of surface residues may profoundly affect the binding properties of the molecule. The more tightly a polypeptide segment is constrained, the less likely it is to bind to any particular target. If it does bind, however, the binding is likely to be tighter and more specific. Thus, it is preferred to select a parental binding domain wherein the parental polypeptide has structure and, thereby in turn, select a structure for the polypeptide analogues of the library, which is constrained within a framework having some degree of rigidity.

Preferably the protein domain that is used as the template or parental domain for generating the library of domain analogues will be a peptide molecule that is a relatively small protein or polypeptide. Small polypeptides offer several advantages over large proteins: First, the mass per binding site is reduced. Highly stable protein domains having low molecular weights, for example, Kunitz domains (~7 kilodaltons, kDa), Kazal domains (~7 kDa), *Cucurbita maxima* trypsin inhibitor (CMTI) domains (~3.5 kDa), and endothelin (~2 kDa), can show much higher binding per gram than do antibodies (150 kDa) or single chain scFv antibodies (30 kDa). Second, the possibility of non-specific binding is reduced because there is less molecular surface available for nonspecific binding. Third, small polypeptides can be engineered to have unique tethering sites in a way that is impracticable for larger proteins or antibodies. For example, small proteins and polypeptides can be engineered to have lysines only at sites suitable for tethering to a chromatography matrix. This is not feasible for antibodies. Fourth, a constrained polypeptide structure is more likely to retain its functionality when transferred (with the structural domain intact) from one framework to another. For instance, the binding domain structure is likely to be transferable from the framework used for presentation in a library, such as displayed on a phage, to an isolated protein removed from the presentation framework or immobilized on a chromatographic substrate.

In specific embodiments, the BLYS binding polypeptides of the invention are immobilized. BLYS binding polypeptide molecules according to the invention may be immobilized, for example, on chromatographic support materials to form efficient BLYS separation or affinity chromatographic media. Immobilized BLYS binding polypeptides of the invention have uses that include, but are not limited to, detecting, isolating or removing BLYS target proteins from solutions. One strategy for generating BLYS binding polypeptide molecules that can be immobilized, for example, on matrices, resins, or supports, involves selecting

appropriate binding domain templates such that BLYS binding polypeptide molecules are generated that have one or more amino acids that may be used to covalently link the BLYS binding polypeptide to a chromatographic resin or substrate to form an affinity resin. Similarly, the N-terminal amino group or the C-terminal carboxyl group of a peptide molecule may be modified by adding a capping group to render it inert or a functional group, which permits linkage to a support medium. For example, the C-terminal carboxyl group of a protein domain may be converted to an amide or a hydrazide (-NH-NH<sub>2</sub>) group for reaction with an aldehyde-functional substrate or other amine-reactive substrate. This technique is preferred. Another preferred modification of BLYS binding polypeptides useful for linking a BLYS binding polypeptide molecule of the invention to a chromatography material is a polypeptide linker comprising, or alternatively consisting of, the amino acid sequence Pro-Gly-Pro-Glu-Gly-Gly-Gly-Lys (SEQ ID NO:13).

In one non-limiting example of a screening procedure to obtain BLYS binding polypeptides encompassed by the invention, the phage in a phage display library are contacted with and allowed to bind a BLYS target protein that is immobilized on a solid support. Those phage that display non-binding polypeptides are separated from those that bind the BLYS target protein. Any of various techniques known in the art may be applied to dissociate the bound phage from the immobilized BLYS protein, and to collect and/or amplify the phage and/or their nucleic acid contents. Using these techniques it is possible to identify a BLYS binding phage that is about 1 in 20 million in the population. Libraries, displaying 10-20 million or more potential binding peptide molecules each, are rapidly screened to find high-affinity BLYS binding polypeptides.

In each round of screening, the diversity of a population falls until only efficient binding polypeptides remain, that is, the process converges. Typically, a phage display library will contain several closely related binding polypeptides (10 to 50 different binding polypeptides out of 10 million). Indications of convergence include increased binding (measured by phage titers) and recovery of closely related sequences. After a first set of binding polypeptide molecules is identified, the sequence information can be used to design other libraries biased for members having additional desired properties, for example, discrimination between different forms of BLYS (e.g., the membrane form and the soluble form of BLYS) and fragments thereof, or discrimination between BLYS and closely related impurities in a feed stream.

Such techniques make it possible not only to screen a large number of potential binding polypeptides, but make it practical to repeat the binding and elution cycles and to build secondary, biased libraries for screening polypeptide analogue-displaying phage that meet specific criteria. Using these techniques, a polypeptide analogue biased library may be screened to reveal members that bind tightly, that is, have high affinity for BLYS target protein, under the screening conditions.

In the present invention target BLYS protein molecules were biotinylated and then bound to streptavidin-coated magnetic particles. Nine phage display libraries of different design were screened for the ability to bind the immobilized BLYS. Each library was characterized by M13 phage displaying variegated peptides of different lengths and overall structure: A library designated TN6/6 ( $2 \times 10^8$  variants) displayed a variegated 12-mer with two internal invariant cyteines to form a hexamer loop structure. A library designated TN7/4 ( $2.3 \times 10^9$  variants) presented a variegated 13-mer having two internal invariant cyteines to form a heptamer loop structure. A library designated TN8/9 ( $5 \times 10^9$  variants) displayed a variegated 14-mer with two internal invariant cyteines to form an octamer loop structure. A library designated TN9/4 ( $3.2 \times 10^9$  variants) presented a variegated 16-mer having two internal invariant cyteines to form a nonamer loop structure. A library designated TN10/9 ( $2.5 \times 10^9$  variants) displayed a variegated 16-mer with two internal invariant cyteines to form a decamer loop structure. A library designated TN12/1 ( $1.4 \times 10^9$  variants) presented a variegated 18-mer having two internal invariant cyteines to form a dodecamer loop structure. A library designated as Substrate Phage Library #2, having a diversity of about  $2 \times 10^8$  amino acid sequences, was designed to include a linear peptide-variegated region in the display polypeptide consisting of 13 consecutive amino acids, and the display polypeptide design allowed any amino acid residue except cysteine to occur at each position. Finally, two commercially available linear phage display libraries were also screened, designated PhD 7 and PhD 12, respectively (New England Biolabs). The PhD 7 library displayed a linear random-sequence 7-mer; the PhD 12 library displayed a random-sequence 12-mer.

BLYS binding phage were isolated and collected from all of the libraries except PhD 7.

After analysis of the sequences isolated from the library screenings, several families of BLYS binding peptides were defined (see, consensus sequences A-G and H-L, above). The

amino acid sequences of the BLyS-binding "hits" from the first rounds of screening are set forth in Tables 1-8 (*infra*).

In order to obtain BLyS binding polypeptides having an even higher affinity for BLyS targets, a specialized library was prepared, i.e., a BLyS affinity maturation library, based on variegation of high affinity exemplars of the PhD 12 library (see Example 6). This library was designed to provide a population enriched with polypeptides likely to show high affinity for BLyS. The selections from this library were performed to eliminate, by prolonged competition with soluble eluants of soluble BLyS or other BLyS binding polypeptides, all but the polypeptides having the highest affinity for BLyS. A large family of high affinity BLyS binding polypeptides was isolated from four rounds of screening the affinity maturation library, and their amino acid sequences appear in Table 14 (*infra*).

BLyS binding polypeptides according to the invention and phage comprising such polypeptides have uses that include, but are not limited to, detecting and isolating BLyS and BLyS-like polypeptides, as described above.

Isolation of BLyS binding polypeptides and their use in accordance with this invention will be further illustrated below. The specific parameters included in the following examples are intended to illustrate the practice of the invention, and they are not presented to in any way limit the scope of the invention.

#### Example 1: Screening of Phage Display Libraries

Streptavidin-coated magnetic beads (Dynal M-280) were chosen for presentation of the target during screening because of their superior binding capacity compared to that of a 96 well plate. The binding capacity of the beads for biotinylated antibodies was 5-10 µg/mg of beads as stated by the manufacturer. For this study and the screening to follow, 5 µg of biotinylated recombinant BLyS (obtained from Human Genome Sciences, Inc.) was allowed for each mg of beads. This amount of biotinylated BLyS represents a 10-fold excess of target, for saturation of the beads. Unbound BLyS was washed away. Bound biotinylated BLyS was confirmed with detection using Mab 16C9 (murine anti-BLyS, Human Genome Sciences) primary antibody and a goat anti-mouse HRP conjugate as the secondary antibody. An irrelevant monoclonal antibody (anti-TNFα) was used to probe a second set of beads to control for nonspecific binding. The color reagent TMB was used and the assay read at OD 630 nm.

Nine libraries, TN6/6, TN7/4, TN8/9, TN9/4, TN10/9, TN12/1, Substrate Phage #2, PhD7, and PhD12, were screened for BLyS binders. The makeup of these libraries was as follows:

The TN6/6 phage display library was composed of recombinant M13 phage displaying variegated peptides with the potential to form loop structures based on a polypeptide template having the structure Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa (SEQ ID NO:14) and providing  $2.0 \times 10^8$  peptide diversity.

The TN7/4 phage display library was composed of recombinant M13 phage displaying variegated peptides with the potential to form loop structures based on a polypeptide template having the structure Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa (SEQ ID NO:15) and providing  $2.3 \times 10^9$  peptide diversity.

The TN8/9 phage display library was composed of recombinant M13 phage displaying variegated peptides with the potential to form loop structures based on a polypeptide template having the structure Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa (SEQ ID NO:16) and providing about  $5 \times 10^9$  peptide diversity.

The TN9/4 phage display library was composed of recombinant M13 phage displaying variegated peptides with the potential to form loop structures based on a polypeptide template having the structure Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa (SEQ ID NO:17) and providing about  $3.2 \times 10^9$  peptide diversity.

The TN10/9 phage display library was composed of recombinant M13 phage displaying variegated peptides with the potential to form loop structures based on a polypeptide template having the structure Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa (SEQ ID NO:18) and providing  $2.5 \times 10^9$  peptide diversity.

The TN12/1 phage display library was composed of recombinant M13 phage displaying variegated peptides with the potential to form loop structures based on a polypeptide template having the structure Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Xaa (SEQ ID NO:19) and providing  $1.4 \times 10^9$  peptide diversity.

Substrate Phage Library #2 was composed of recombinant M13 phage displaying a polypeptide insert of approximately 80 amino acids, having two streptavidin binding domains, a linear variegated segment of thirteen amino acids where all amino acids except Cys were



permitted at each position, and a Factor Xa cleavage site, linked together with peptide linkers. This library provided a diversity of  $2 \times 10^8$  display polypeptides.

Libraries PhD7 and PhD12 were composed of recombinant M13 phage displaying randomized linear seven- and twelve-amino acid peptides, obtained commercially from New England Biolabs.

Prior to each round of screening, phage libraries or phage library pools were depleted of phage capable of binding the streptavidin beads by sequentially adding the libraries to 5 separate aliquots of streptavidin beads and allowing them to bind for 10 minutes. The depleted libraries were added to biotinylated BLYS on streptavidin magnetic beads and allowed to bind for 1 hour at room temperature. For round 1 of the screening, all the libraries were kept separate except PhD7 and PhD12 which were pooled from the beginning. After binding, the beads were washed 7 times and bound phage were incubated for 10 minutes with citrate buffered saline at pH 2.0 to elute. The eluted phage were neutralized with 2 M Tris-HCl pH 8.0 and allowed to infect *E. coli* XL-1 Blue MRF'. The infected cells were spread on a large agar plate and standard phage techniques known in the art were used to produce the starting material for the next round. For each round of screening the fraction of input recovered was calculated for each library (library pool). This is equal to the number of phage recovered divided by the number on starting phage. No further rounds of screening were done after the fraction of input recovered reached  $1 \times 10^{-2}$  total phage. Pool A for round 2 of screening was a mixture of TN6/6, TN7/4 and TN8/9 round 1 outputs. Pool B for round 2 was a mixture of TN9/4, TN10/9, and TN12/1 round 1 outputs. After round 2 on Pool A and Pool B the fraction of input recovered was equal to or greater than  $1 \times 10^{-2}$  and no further rounds were done. For the Substrate Phage Library #2 and the PhD pool a third round of screening was required.

At the conclusion of screening individual phage isolates were randomly selected and tested by ELISA for binding to BLYS. The same isolates were submitted for DNA sequence analysis to identify the nucleotide and deduced amino acid sequence of the displayed peptide. Isolates were also tested for their ability to bind to recombinant BLYS in feed streams of CHO supernatant and Sf9 supernatant (supplied by Human Genome Sciences, Inc.).

Each isolate was tested for binding to BLYS by standard ELISA techniques where bound phage were detected with a monoclonal anti-phage antibody/HRP conjugate. Approximately 90% of the isolates from the TN libraries Pool A and Pool B had binding signals on BLYS

ranging from 3x to 12x above the background binding on streptavidin alone. Isolates from the Substrate Phage Library showed similar but slightly lower binding signals.

To assess the ability of the BLYS binding polypeptides to recognize the BLYS target in potential process feed streams, phage binding was determined in two feed streams: CHO and Sf9 supernatants spiked with BLYS protein. Phage were allowed to bind to BLYS in either CHO supernatant or Sf9 supernatant rather than the standard conditions of PBS plus Tween. All other wash steps were the same as the standard ELISA conditions. The binding of BLYS binding polypeptides to BLYS in PBS+Tween, CHO supernatant, and Sf9 supernatant binding was very similar under all conditions. Several BLYS binding polypeptide isolates demonstrated reduced binding of up to 50% in the CHO supernatant. Isolates of BLYS binding polypeptides binding to BLYS in the Sf9 supernatant was not significantly different from binding under the standard conditions. The PhD and Substrate Phage Library isolates were also compared in the feed streams. Remarkably, several of these isolates exhibited greater binding in the feed streams than under the standard conditions.

Amino acid sequences of the displayed peptides were derived from sequencing the phage isolate DNA inserts. Sequence data from the phage isolates were grouped by library and sorted according to the degree of similarity. The BLYS binding phage isolate peptides are shown in Tables 1-8 below. These peptides represent the translation of the DNA sequences across the varied regions of the genes encoding the phage display fusion/peptide.

Table 1

TN6/6 Library BLyS-binding Sequences

	<u>Phage Isolate</u>	<u>Amino Acid Sequence</u>	<u>SEQ ID NO:</u>
5	453-01-B06	HLRCWSTNCRYD	20
	453-01-A04	VMDCLINRCDTV	21

Table 2

TN7/4 Library BLyS-binding Sequences

	<u>Phage Isolate</u>	<u>Amino Acid Sequence</u>	<u>SEQ ID NO:</u>
10	453-01-B04	KSKCFFPWECQQA	22
	453-01-D11	AMKCYFPWECANG	23
	453-01-A05	NVACYFPWECHHP	24
15	453-01-D01	NAPCYFPWECFSI	25
	453-01-D03	SVNCWFPWECVGN	26
	453-01-A08	KEPCYFYWECAVS	27

Table 3

## TN8/9 Library BLyS-binding Sequences

	Phage Isolate	Amino Acid Sequence	SEQ ID NO:
	453-01-D04	DTNCDLLTKMCGPQ	28
5	453-01-C06	GTPCDLLTKLCLLW	29
	453-01-D10	MSECDLLTKICLMG	30
	453-01-B07	VPFCDLLTKHCFEA	31
	453-01-B09	VPFCDLLTKHCFEA	32
	453-01-C02	WSACDLLTKQCVQV	33
10	453-01-A06	-DGCDELTKICGMK	34
	453-01-B03	KSWCDELTKVCFDP	35
	453-01-B11	KWMCDELTKQCQYV	36
	453-01-A02	MKYCDELTKICVGW	37
	453-01-B05	YFQCDELTKMCWQK	38
15	453-01-A11	AMHCDKLTKHCKFH	39
	453-01-A03	VPYCDKLTIKCQW-	40
	453-01-A07	EVFCDVLTKVCFHD	41
	453-01-C09	KPKCDVLTKMCDWL	42
	453-01-B02	TQHCDVLTKQCFTI	43
	453-01-C01	GHFCDRLTKYCFEP	44
	453-01-A09	HIQCDRLTKSCLSV	45
	453-01-D05	IKACDILTKVCWPP	46
	453-01-A01	QFDCDPLTKYCGEF	47
	453-01-C07	KMYCDHLTGycWPE	48
	453-01-C11	MQSCDILTGycFKR	49
	453-01-D12	GPWCDILTGfCLAQ	50
	453-01-C04	SVRCDLLTGWCPVW	51
	453-01-B10	PADCDPLTNICFWK	52
	453-01-D02	TNVC DPLTNVCFMN	53
	453-01-C05	EHWCDDLTHLCFRL	54
	453-01-D08	GYWCDVLTNNCWKI	55
	453-01-C10	LYNCDYLTRLCFEP	56
	453-01-C08	HVDCLLHPKACYKY	57
	453-01-D07	VQDCLLHPKACQMQ	58
35	453-01-D09	KFDCLLKPMFCSNH	59
	453-01-C12	FADCLIHPKsckPL	60
	453-01-D06	HGNCYPFPWECESK	61
	453-01-B01	MIIVLLLLRFAISR	62
	453-01-A12	SLLVIFLLIGAGSL	63

Table 4

## TN9/4 Library BLyS-binding Sequences

	Phage Isolate	Amino Acid Sequence	SEQ ID NO:
	453-01-G06	FHPCDMLTGIWCQPN	64
5	453-01-H01	SKRCDLLTKMWCETE	65
	453-01-F02	TKFCDRLTMPKCVWK	66
	453-01-E03	NTFCPDPLTGRCVNP	67
	453-01-E11	DWTCDPLFHRECIFE	68
	453-01-H09	PQPCDLLFEKKCSIK	69
10	453-01-H02	RWHCDMLINPSCLPD	70
	453-01-E04	KIQCDIVNLSSCVYP	71
	453-01-G11	LNACDIVHPNYCSGM	72
	453-01-F01	AKACSIIVNLESCEYL	73
	453-01-H06	RQACSIITPWGCPPI	74
15	453-01-F10	ADNCTVATLDFCYWT	75
	453-01-E05	KPECNITKPQFCFGE	76

Table 5

## TN10 Library BLyS-binding Sequences

	Phage Isolate	Amino Acid Sequence	SEQ ID NO:
	453-01-H07	-NNCQWDELTSMDPF	77
	453-01-F05	SRLCHMDELTHVCVHF	78
	453-01-F09	SRPCQIDELTKACFYN	79
	453-01-G09	DRVCKLDFLTYNCLNH	80
	453-01-F04	HSNCIMDLLTNRCFYD	81
	453-01-H03	PFNCFHDPLTGLCLHS	82
	453-01-F03	YDSCTYDRLTKQCYPS	83
	453-01-F07	FHDCMYDALLGYCLPY	84
	453-01-G08	NRSCDPLTRPKSCGL	85
	453-01-G04	LSNCDWDDLIRQCLHD	86
	453-01-E01	FWDCLFHPNSRYCVLS	87
	453-01-E10	SRDCLLSPAMAWCGLD	88

Table 6

## TN12/1 Library BLyS-binding Sequences

	Phage Isolate	Amino Acid Sequence	SEQ ID NO:
	453-01-H05	GGNCYTDSLTKLHFCMGD	89
5	453-01-H04	--MCPRDPLTKAKLCNWH	90
	453-01-G03	PNQCQDDLTQWYSCHYH	91
	453-01-F11	FDMCFDALTKQNFYCRFH	92
	453-01-F06	RNMCVDRLTKLQHGCCEGA	93
	453-01-G07	DPECLTSFDRLTKMCWPW	94
10	453-01-H11	DDECHYDYLTHYMRCDYR	95
	453-01-G05	FGGCNIDLLTNTMMCHRN	96
	453-01-G10	HGPCYWDELTMQWHCNHH	97
	453-01-H12	GAMCVDLLTYTFRPCMYA	98
	453-01-E07	SNKCWDELTHAWAECGRF	99
15	453-01-E09	RPVCYKGYDILTTQCMPW	100
	453-01-G01	PSRCWFDLLFNKFVCKRN	101
	453-01-H08	RSGCVYDMLLMTMYCPSN	102
	453-01-H10	SNRCEGDQLMRPPSCRHL	103
	453-01-F08	YRMCWWDDLLRGFVCDHF	104
	453-01-E06	HDGCYDELLYRWTRCEHR	105
	453-01-E08	WAWCFDELVQRYFTCFDH	106
	453-01-E02	LPECRQYFPWEKQVCSYW	107

Table 7

## PhD 12 Library BLyS-binding Sequences

	Phage Isolate	Amino Acid Sequence	SEQ ID NO:
	453-02-B05	VHYDSLTKMWTR	108
5	453-02-D09	FTDPLTKMSLHS	109
	453-02-C12	GYDVLTKLYFVP	110
	453-02-A05	YYDRLTKLYSSM	111
	453-02-B06	L?KDPLTKLYIS	112
	453-02-A04	GYDVLTKL?FVP	113
10	453-02-B03	RLYDPLTKLVLS	114
	453-02-B01	MFDPLTKIAFPA	115
	453-02-D04	FYDSLTKTNLRD	116
	453-02-B02	GIYDKLTRAWLP	117
	453-02-B08	KYDPLTRAR?PL	118
15	453-02-D06	YIDQLTRLSSLPS	119
	453-02-A09	HqTFDILTRLHF	120
	453-02-B04	WQFDVLTRS WTP	121
	453-02-A02	GAAYDHLTRTWL	122
	453-02-D05	YFDQLTHLSIKK	123
	453-02-A06	AWDPLTMLVLPW	124
	453-02-D03	ALWMDPLTGLAF	125
	453-02-B12	WQFDVLT?SWTP	126
	453-02-A01	WTDPLTHMEIYH	127
	453-02-C04	WTDSLTGLWFPD	128
	453-02-C05	YTDPLTGIV?PF	129
	453-02-D08	YWDKLTMLHLGV	130
	453-02-D02	YYDFLTRTVLPS	131
	453-02-A03	RLDPLSKNDFPR	132
	453-02-A11	LRYPDLLKS?IY	133
	453-02-D07	LRYPDLLKSYIY	134
	453-02-A07	YFDQFTHLSIKK	135
	453-02-C08	YFDQ?THLSIKK	136

Table 8

## Substrate Phage Library BLyS-binding Sequences

	Phage Isolate	Amino Acid Sequence	SEQ ID NO:
	453-02-E04	EHYYTDPLTGARI	137
5	453-02-F01	EHY?TDPLTGARI	138
	453-02-E09	EHYSTDPLTGARI	139
	453-02-E07	EHYYTDPL?G?RI	140
	453-02-G05	EHYYTDPL?G?R?	141
	453-02-G09	EHYYTDPL?GAR?	142
10	453-02-E06	EH?YTDPLNGAR?	143
	453-02-E05	EHYYNDPLNGAR?	144
	453-02-F04	?H?YNDPLNGAR?	145
	453-02-G07	KPYYPDITKMTHH	146
	453-02-F06	KPYYPDITKMSHH	147
15	453-02-E08	KPYYPDISKMTHH	148
	453-02-G08	KP??DPISKMTHH	149
	453-02-E01	QIGYDELTKAWVT	150
	453-02-G02	QLGYDELTKAWVT	151
	453-02-H06	KIDEL?MQNIIIW	152
	453-02-F08	DHTDPLIQGLTKR	153
	453-02-H01	WHDPLKMHMFHHE	154
	453-02-F03	KHIDMETGLILQN	155
	453-02-G03	MQVDPETGLKYE	156
	453-02-E03	?LDQHVN???YQS	157
	453-02-F10	E???T??LTGAR?	158
	453-02-F02	GPYNI?RL?GER?	159
	453-02-E02	HIKMLHQGSFVGV	160
	453-02-H08	HPTNT??HQ?VYS	161
	453-02-H05	HRGQV??LNGMv?	162
	?= amino acid unknown (all tables)		
	lower case = amino acid identity probable but not completely characterized		

A small number of phage isolates were selected for further characterization based upon several criteria: the strength of the ELISA signal (i.e.,  $OD_{630} \geq 0.8$  after 10 min.), the number of times the identical sequence was found, and the presence of a recurrent sequence motif. Some characteristics of the phage isolates that were selected are shown below. Sequence motifs found multiple times in the isolates are underlined.



	Isolate	#	ELISA signal	Sequence	SEQ ID NO:
	TN7-01-A08	1	1.2	KEPCYFYWECAVS	27
5	TN7-01-D11	2	1.0	AMKCYFPWECANG	23
	TN7-01-B04	2	0.8	KSKCFPPWECQQA	22
	TN8-01-C08	2	1.2	HVDCLLHPKACYKY	57
	TN8-01-B07	2	1.4	VPFCDLLTKHCFEA	31
	TN9-01-G06	1	1.2	FHPCDMLTGIWCQPN	64
10	TN9-01-G11	1	0.8	LNACDIVHPNYCSGM	72
	TN10-01-F05	1	1.0	SRLCHMDELTHVCVHF	78
	TN12-01-H05	1	1.0	GGNCYTDSLTKLHFCMGD	89
	PhD-02-B02	4	0.6	GIYDKLTRAWLP	117
	PhD-02-B05	9	0.6	VHYDSLTKMWTR	108
	PhD-02-C12	3	0.8	GYDVLTKLYFVP	110
	PhD-02-D05	3	0.8	YFDQLTHLSIKK	123
	PhD-02-C04	1	1.0	WTDSLTGLWFPD	128

Various release conditions (see below) were tested, in order to discover possible elution conditions where the BLyS binding polypeptides could be used as affinity ligands for BLyS purification. For release studies, a constant number of phage were applied to wells containing biotinylated BLyS immobilized on streptavidin. After allowing the phage to bind, each phage isolate was then "eluted" from the well with two five-minute washes using various buffers. Wells were washed with standard wash buffer, and bound phage were detected with a standard phage ELISA. Elution conditions were selected based on low pH release, which was the mechanism employed during screening, and alternative elution conditions based on conditions where the BLyS product was known to be stable for at least several hours. The various elution conditions were: PBS pH 7.0, citrate buffered saline pH 5.0, citrate buffered saline pH 3.0, citrate buffered saline pH 2.0, 1 M Guanidine pH 7.0, and 1 M urea pH 7.0. Binding of several of the isolates was reduced under the standard conditions (PBS pH 7.0). This may have occurred because these experiments were performed with concentrated phage preparations rather than the overnight bacterial supernatants used for all previous experiments. It is believed that the

polyethylene glycol used to concentrate the phage interfered with the binding of these isolates. For the purposes of these release studies, the phage isolates selected had a starting signal of 0.5 OD 630 nm or greater. Overall, 1 M urea, pH 7.0 appeared to be the best buffer to release bound phage.

5

#### Example 2: Immobilization of BLYS Binding Polypeptides on Sepharose-4FF Beads

On the basis of the above results, six display phage sequences were chosen for further study:

10 TN7-01-A08 (SEQ ID NO:27), TN8-01-B07 (SEQ ID NO:31), TN10-01-F05 (SEQ ID NO:78), TN12-01-H05 (SEQ ID NO:89), PhD-02-C04 (SEQ ID NO:128), and PhD-02-C12 (SEQ ID NO:110). In order to develop a suitable BLYS affinity ligand, the identified display peptides were synthesized to order by a commercial vendor, with slight modifications:

Two amino acids of leader were added to each binding peptide at the N-terminus, in order to avoid leaving a free amine at the first amino acid of the sequence corresponding to the variegated region of the phage display template; the N-terminus was acetylated to prevent immobilization of the peptide to the chromatographic matrix through that position; a C-terminal linker was added (i.e., -PGPEGGGK; SEQ ID NO:13); and any internal lysines in the peptide were blocked with the group: ivDde (i.e., 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methyl butyl-L-lysine). This group was intact on the finished synthesized peptides and was removed after immobilization or fluorescein labeling. As an alternative modification, peptides with internal lysines were also synthesized with C-terminal hydrazide functional groups, which could be immobilized onto activated aldehyde chromatographic media.

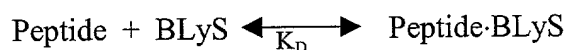
25 The peptides were immobilized onto NHS-activated SEPHAROSE-4 Fast Flow agarose media (Pharmacia) at ligand densities targeted to 2  $\mu\text{mol/ml}$ . Actual ligand densities of peptides on the media ranged from 0.76  $\mu\text{mol/ml}$  to 1.98  $\mu\text{mol/ml}$ , as determined by amino acid analysis of immobilized peptide. All but one peptide was immobilized in aqueous conditions of 100mM  $\text{KH}_2\text{PO}_4$ /150mM NaCl/0.05% Tween 20, pH 7.5. For solubility reasons, the peptide DX217 (see, Table 9, below) was immobilized in 30% dimethyl formamide(DMF)/100mM  $\text{KH}_2\text{PO}_4$ /150mM NaCl/0.05% Tween 20. pH 7.5. Immobilization reactions were allowed to proceed for 2 hours at ambient temperature, followed by brief washing with pH 7.5 buffer. The Fast Flow SEPHAROSE media was then allowed to tumble at ambient temperature overnight to

hydrolyze remaining NHS esters after which the media was washed to remove any unbound peptide. A solution of 2% hydrazine/DMF was used to de-block ligands containing ivDde-lysine. Media was then further washed with aqueous buffers and stored at 4°C until packed into columns. Table 9 shows the sequences of the synthesized peptides and their measured densities on the agarose media.

Table 9: BLYS Binding Peptides Synthesized as Affinity Ligands			
Peptide Name	Isolate source	Sequence (potential disulfide loop underlined)	SEQ ID NO:
DX212	01-A08	Ac-AGKEPCYFYWECAVSGPGPEGGGK	163
DX214	01-B07	Ac-AGVPFCDLLTKHCFEAGPGPEGGGK	164
DX216	01-F-5	Ac-GSSRLCHMDELTHVCVHFAPPGPEGGGK	165
DX217	01-H05	Ac-GDGGNCYTDSLTKLHFCMGDEPGPEGGGK	166
DX219	02-C12	Ac-GYDVLTKLYFVPGPGPEGGGK	167
DX221	02-C04	Ac-WTDSLTLGLWFPDGGPGPEGGGK	168

#### BLYS-Ligand Affinity Determination (Overview of Procedure)

Dissociation constants between the synthetic peptides and BLYS (free in solution) were measured by fluorescence anisotropy (FA). In these experiments, the concentration of the fluorescein-labeled peptide is held constant and the BLYS protein concentration was varied. The observed change in anisotropy is fit to the following equation via nonlinear regression to obtain the apparent  $K_D$ .



$$r_{obs} = r_{free} + (r_{bound} - r_{free}) \frac{(K_D + BLYS + P) - \sqrt{(K_D + BLYS + P)^2 - 4 \cdot BLYS \cdot P}}{2 \cdot P}$$

where:

$r_{\text{obs}}$  = observed anisotropy,  $r_{\text{free}}$  = anisotropy of free peptide,  $r_{\text{bound}}$  = anisotropy of bound peptide,  $K_D$  = dissociation constant, BLYS = total BLYS concentration, and P = total fluorescein labeled peptide concentration.

Binding reactions containing 50 nM fluorescein-labeled peptide and a varied concentration of BLYS in a volume between 10 and 20  $\mu\text{L}$  per well were performed in 384 well microplates. Reactions were assayed using a Tecan Polarion fluorescence polarization plate reader. Cross-competition studies between peptides were performed using 50 nM fluorescein-labeled peptide and 1-2  $\mu\text{M}$  BLYS in the presence and absence of 100  $\mu\text{M}$  unlabeled peptide. The influence of pH on the observed  $K_D$  was investigated at pH 6.0 using the primary binding buffer [15 mM sodium citrate, 120 mM NaCl, 0.01% Tween 20] and at pH 9.0 using 200mM sodium bicarbonate, 125 mM sodium chloride. Other buffers in which dissociation constants of BLYS Binding polypeptides were determined include: [pH 6.0, 0.01% Tween], [pH 6.0, 0.1% gelatin], [pH5.0, 0.01% Tween], [pH9.0, 0.1% Tween], [pH6.0, 15% ethylene glycol, 0.01 % Tween], [pH5.0, 15% ethylene glycol, 0.01 % Tween], and [pH9.0, 15% ethylene glycol, 0.01 % Tween]. All six of the peptides (DX212, DX214, DX216, DX217, DX219, and DX221) bound BLYS in solution with approximately the same affinity ( $K_D = 0.4 - 3 \mu\text{M}$ ). Cross-competition studies demonstrated that all peptides compete with each other for BLYS binding, which suggests that they all bind to the same site on BLYS.

### Example 3: Chromatographic Screening of Immobilized BLYS Binding Polypeptides

A reversed phase analytical assay was used in the assessment of the chromatographic performance of the six affinity media.

The six affinity media (BLYS binding polypeptides bound to SEPHAROSE 4 Fast Flow) and a control column, (hydrolyzed NHS-SEPHAROSE 4 Fast Flow) were packed into 3 x 50 mm glass Omnifit columns (350  $\mu\text{L}$ ). All columns were tested at 200  $\mu\text{L}/\text{min}$  (170cm/hr) using a Watson/Marlow 101 ru peristaltic pump. This setup allowed free use of numerous wash, protein and elution conditions. Detection was made using a Waters 2487 UV/VIS detector at 214 nm and 280 nm connected to a Waters Millennium workstation.

Initial screens with purified BLYS at 30  $\mu\text{g}/\text{ml}$  in PBS/0.01%Tween 20, pH 7.2 showed 65% recovery in the flow-through of the control column (1ml, 30  $\mu\text{g}$  total). However, it was immediately clear that all of the columns, when tested in the same manner, bound BLYS

quantitatively from solution, but did not release the protein. The columns were then tested with an array of possible elution conditions and monitored at 214nm and 280nm for release of BLYS. Fractions indicating possible BLYS elution based on UV absorbance were collected and analyzed by reversed phase chromatography for confirmation. Conditions tested are shown in Table 10 (below).

Table 10: Chromatographic Elution Conditions, BLYS Affinity Media			
Chaotropic Salts	Other Salts and pH	Other Buffers and pH	Organic Solvents
1M urea, pH 7	1 M NaCl, pH 7 and 5	30 mM H <sub>3</sub> PO <sub>4</sub> , pH 2	50% ethylene glycol, pH 4
2M urea	2 M NaCl, pH 7 and 5	200 mM NaCO <sub>3</sub> , pH 10	50% ethylene glycol, pH 7
4M urea, pH 7	2 M MgCl <sub>2</sub> , pH 7.6	50-100 mM NaOAc, pH 5	20% ethanol
2M guanidine, pH 7	2 M CaCl <sub>2</sub> , pH 6	2M imidazole, pH 6	50% ethanol
	1 M citrate, pH 6	0.6M Histidine, pH 6	18% butanol
		1M Arginine, pH6	30% glycerol
		100mM EGTA, EDTA	
		1 M sorbitol, pH 7	

Columns made with DX214, DX216, DX217, DX219 and DX221 affinity media would only release BLYS in the presence of pH 2 buffer (30 mM H<sub>3</sub>PO<sub>4</sub>/150mM NaCl, pH 2). Recoveries from these columns ranged between 30 % and 65%. In contrast, DX212 affinity media released BLYS with good recoveries on elution with 50% ethylene glycol, pH 4 (50% ethylene glycol/100mM sodium acetate, pH 4) or with 50% ethylene glycol, pH 5 (50% ethylene glycol/100mM sodium acetate, pH 5).

To determine if the elution conditions were effective at maintaining BLYS in its native trimeric form, size exclusion chromatography (SEC) was used to assay native BLYS and BLYS exposed to both pH 2 and 50% ethylene glycol/100 mM sodium acetate, pH 5. SEC analysis of BLYS following incubation at pH 2 revealed the presence of two new peaks, corresponding in size to apparent multimer and monomer forms, with no evidence of the native trimer. Incubation in 50% ethylene glycol resulted in 16% multimer, but otherwise maintained trimer. Later SEC results on material eluted from the DX212 column with 50% ethylene glycol (pH 5.0) did not show the multimer.

#### Example 4: Capture of BLYS from Cell Culture Supernatants

The DX212, DX219, and DX214 affinity columns were tested for their ability to purify BLYS from cell culture supernatants. BLYS, at approximately 40 µg/ml, was spiked into thawed cell culture supernatants from CHO and Sf9 cell lines. Approximately 100 µg BLYS (2.5 ml total) was loaded onto each column. Levels of BLYS in Sf9 flow-through samples could not be determined in the reversed phase HPLC assay. BLYS was eluted with elution buffer [50% ethylene glycol, 100mM NaAc, pH5.0]. BLYS protein recovery from these experiments ranged from 29.4% to complete recovery, with purities ranging from 76% to 96.5%.

#### Example 5: Synthesis of Further BLYS Binding Peptides

Once a promising BLYS binding polypeptide has been isolated, improvements to that polypeptide can be made by changing, adding or removing individual or multiple amino acid residues from the polypeptide. Amino acid substitutions can be conservative or non conservative. Conservative amino acids exchanges include, for example, the exchange of aromatic residues (e.g., phenylalanine, tryptophan, and tyrosine) for one another, the exchange of hydrophobic residues (e.g, leucine, isoleucine, and valine) for one another , the exchange of polar residues (e.g., glutamine and asparagine) for one another, the exchange of acidic residues (e.g., arginine, lysine, and histidine) for one another, and the exchange of small residues (e.g., alanine, serine, threonine, methionine, and glycine) for one another, the exchange of aromatic residues for one another. Additionally, nonclassical amino acids, chemical amino acid analogs, or chemically modified classical amino acids can be introduced as a substitution or addition to a BLYS binding polypeptide of the invention. Non-classical amino acids include, but are not limited to, the D-isomers of the common amino acids, 2,4-diaminobutyric acid (Dbu), 4-aminobutyric acid (bAbu), 2-aminobutyric acid (Abu), 6-amino hexanoic acid (epsilon-Ahx), 2-aminoisobutyric acid (Aib), 3-aminoisobutyric acid (bAib), 3-aminopropanoic acid (bAla), ornithine (Orn), norleucine (Nle), norvaline (Nva), 3-hydroxyproline (3Hyp), 4-hydroxyproline (4Hyp), sarcosine (MeGly), citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general. By way of example, four modified peptides based on the DX212 sequence have been designed:

1. Ac-AGK(Ac)EPCYFYWECAVSGPGPEGGGK (SEQ ID NO: 169) -- internal lysine side chain acetylated;
2. Ac-AGREPCYFYWECAVSGPGPEGGGK (SEQ ID NO: 170) -- arginine substitution;
3. Ac-AGQEPCYFYWECAVSGPGPEGGGK (SEQ ID NO: 171) -- glutamine substitution;
- 5 4. Ac-AGNleEPCYFYWECAVSGPGPEGGGK (SEQ ID NO: 172) -- norleucine substitution.

#### Example 6: Affinity Maturation of BLYS Binding Polypeptides

In order to identify high affinity BLYS-binding polypeptides, a BLYS Affinity Maturation Library (BAML) was designed around a 14-mer linear peptide template sequence having fixed amino acid residues at 5 of the 14 positions. 3 of the 5 fixed residues corresponded to a highly conserved DxLT tetrapeptide amino acid motif (SEQ ID NO:446) isolated from both the constrained and linear peptide libraries. The design of the 14-mer allowed for some amino acid variation at each of the remaining 9 positions, however, preference was given for a particular amino acid at each of these positions. Analysis of binding affinity of the newly isolated peptides for BLYS was evaluated by direct and indirect phage ELISA and fluorescence anisotropy.

BAML was designed on a 14-mer linear (non-constrained) template peptide sequence having fixed residues at positions 1 (Ala), 5 (Asp), 7 (Leu), 8 (Thr), and 10 (Leu). The amino acid sequence of positions 3-14 in the BAML template most closely resembles a binding polypeptide isolated from the PhD 12 linear polypeptide library (see Table 7, *supra*). Residues at position 1 (fixed Ala) and position 2 (variable) were included to extend the length and presentation of the BLYS-binding sequence. Positions 5-8 correspond to the DxLT motif found in peptide isolates from both the constrained and linear peptide libraries (see Tables 1-8, *supra*). Since hydrophobic amino acids (L, M, I, A, and G) were found at position 10 in 85% of the original isolates, a Leu residue, occurring in 42% of the isolates, was fixed at that position in the BAML template peptide.

Table 11 shows the design of the 14-mer BAML template sequence.

Table 11: BAML template sequence (14-mer)														SEQ ID NO:
amino acid position														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	
A	n	w	y	D	s	L	T	k	L	w	l	p	d	184

Referring to Table 11, the upper case letters indicate the fixed residues at positions 1, 5, 7, 8, and 10 of the template. Lower case letters designate preferred amino acids at those positions, however the design of the variegated DNA template encoding the 14-mer allows for some sequence variation at these positions.

5 Table 12 shows the design of the variegated DNA template used to generate the BAML peptides.

Table 12: BAML DNA template sequence (14-mer)													
codon position													
1	2	3	4	5	6	7	8	9	10	11	12	13	14
codons*													
GCT	eez	zjj	zez	GAT	zqz	CTT	ACT	eej	CTC	zjj	qzz	qqz	jez

\* The sequence of codons is SEQ ID NO:185.

Referring to Table 12, the nucleotide coding sequences for the fixed amino acids in the BAML 14-mer template are shown in upper case letters. The letters "e", "j", "q", and "z" in the variegated DNA template each represent a particular mixture of nucleoside bases present in the input dNTPs for each position:

j = 79% guanine, 7% cytosine, 7% adenine, 7% thymine

q = 7% guanine, 79% cytosine, 7% adenine, 7% thymine

e = 7% guanine, 7% cytosine, 79% adenine, 7% thymine

z = 7% guanine, 7% cytosine, 7% adenine, 79% thymine.

The codons of the DNA template were designed to skew the encoded variable amino acid toward the preferred amino acid at each position shown in SEQ ID NO:184 (Table 11, lower case). Later sequencing of phage isolates showed that, at any particular position, preferred residues occurred at a frequency of from 44% to 70%.

Synthetic DNA sequences fitting the DNA template were amplified by large scale PCR. The amplified DNAs were restriction digested for insertion into a M13 phage expression vector (MANP vector, Dyax Corp., Cambridge, MA), and vectors bearing the inserts were used to transform M13 phage by electroporation, to produce the BAML.

25 Recombinant phage were collected and purified by PEG precipitation and titered. A total of  $3.2 \times 10^{13}$  PFU were amplified in BAML from  $1.6 \times 10^9$  transformants.



## Screening BAML

As outlined in Table 13, a two-step competition method, starting with the original BAML library, was used over 4 rounds of screening to isolate the highest affinity BLYS-binding polypeptides from the BAML. Prior to screening, the amplified BAML was contacted with Seradyn streptavidin-coated magnetic beads (MG-SA, Seradyn, Indianapolis, IN), to remove bead- and streptavidin-binding phage.

For screening BAML, phage were incubated in solution with biotinylated BLYS (b-BLYS) in 200  $\mu$ l PBS, pH 7.4, Tween-20 (0.1%), to form phage/b-BLYS binding complexes. For the first competition step, unlabeled BLYS (1-2  $\mu$ M) was added to the phage/b-BLYS binding complex mixture in solution and incubated for 1-20 hrs. (See Table 13.) The phage/b-BLYS complexes remaining in solution after incubation with unlabeled BLYS were captured by brief (10 min. on rotator) incubation with MG-SA streptavidin beads (50  $\mu$ l). After capture of the phage/b-BLYS complexes on streptavidin beads, the unbound fraction was removed and beads were washed 15-20 times with 1 ml PBS-Tween 20 prior to the second competition step. The phage/unlabeled BLYS complexes from the round 1 competition step only, were collected and used as a fraction of the input phage for the second round of screening along with the bead-captured phage/b-BLYS complexes, however, in each subsequent round of screening only the bead-associated phage were collected after the first competition step for further screening, and the phage/unlabeled BLYS complexes were discarded.

For the second competition step, the competitor peptide was a polypeptide (DX221; SEQ ID NO:168) based on a BLYS-binding polypeptide isolated from the PhD 12 library in the initial screenings described above. The phage/b-BLYS complexes bound to streptavidin, collected after the first competition incubation step, were serially diluted with 50  $\mu$ M DX221 BLYS-binding peptide ( $K_D = 3 \mu$ M) in 300  $\mu$ l PBS-Tween-20 (0.1%). A series of short incubations (3-4 per round, for 1 hour) of the phage/b-BLYS complexes with DX221 followed by a final incubation of from overnight (O/N, for rounds 1, 2, and 4) to 3 days (for round 3). (See Table 13.) The second competition step in round 4 included an incubation with 67 nM BLYS for 1 hour prior to incubation with DX221. The streptavidin bead-associated phage/b-BLYS binding complexes remaining after the DX221 competition step in round 4 were collected for further analysis.

Table 13: BLYS affinity maturation library (BAML) screening conditions

Screening Round	Input phage <sup>1</sup>	b-BLYS <sup>2</sup>	First Competition Incubation Time (hrs)	Competitor (BLYS)	Second Competition Incubation Time (hrs)	Peptide Elutions
1	1.5x10 <sup>11</sup>	100nM	2	2μM	1	50 μM DX221, 4 × 1hr, then O/N
2	2x10 <sup>10</sup>	100nM	1	1μM	20	50 μM DX221, 3 × 1hr, then O/N
3	6.5x10 <sup>10</sup>	100pM	16	1μM	3	50 μM DX221, 4 × 1hr, then 3 days
4	6.0x10 <sup>10</sup>	10pM	16	1μM	2	67 nM BLYS, 1hr; 50 μM DX221 + 67 nM BLYS 3 × 1hr, O/N, then add'l 4hrs

<sup>1</sup> Input phage for round 1 was original BAML; for round 2 was amplified output phage from overnight (final) peptide elution and bead-associated phage from round 1; for round 3 was amplified bead-associated output phage from round 2; and for round 4 was amplified bead-associated output phage from round 3. All amplified phage samples were pre-cleared on streptavidin beads before incubation with biotin-BLYS in solution.

<sup>2</sup> b-BLYS = biotinylated BLYS

### ELISA Analysis

Approximately four hundred BAML isolates from rounds 2, 3 and 4 of the above screening were analyzed by direct and indirect phage ELISA assays.

For indirect phage ELISA, Immulon-2HB plates (Dynex Technologies, Inc., Chantilly, VA) were coated with 100 μl of 1 μg/ml Immunopure streptavidin (Pierce, Rockford, IL) diluted in PBS. 100 μl of a series of 10-fold dilutions of b-BLYS (0-0.1 μg/ml in PBS) were immobilized in the streptavidin-coated wells (1hr, 37°C). After washing, 1-25 μl of overnight culture of *E. coli* infected with the individual phage plaques were added to the appropriate wells and incubated for 1 hour, followed by 10 washes with PBS-Tween-20. Anti-M13 antibody

conjugated to horseradish peroxidase (1:10,000 in PBS-Tween-20) was added to the wells (30 min., room temperature), the color reagent TMB was used and the plates read at OD 630 nm.

Individual phage isolates binding to immobilized BLYS were sequenced and the sequences analyzed. The unique sequences of the BAML BLYS-binding 14-mer display peptides are shown in Table 14.

Analysis of the peptides reveals a significant sequence "collapse" around one motif: W<sub>3</sub>YDPLTKLWL<sub>12</sub> (SEQ ID NO:436) (subscripts indicate amino acid position in the 14-mer display peptide sequence). This most numerous core motif includes the four fixed residues from the original BAML template, i.e., Asp (D) at position 5, Leu (L) at position 7, Thr (T) at position 8, and Leu (L) at position 10. In addition, 5 of the 6 preferred residues from the original BAML template sequence were included in this motif (see Table 11).

73% (143 of 197) of the round 4 isolates included this core motif (SEQ ID NO:436). Single residue substitutions within the 10-mer core motif centered on positions 4 (Y → F) and 12 (L → F, I, or V), with the substitutions at position 12 being alternative hydrophobic residues for Leu.

For the three remaining variable positions (i.e., 2, 13, and 14), selection was not as stringent, although some preferences were apparent, being either built into the library or persisting through rounds of selection. For example, in round 4 isolates, 51% included Asn at position 2; 77% included Pro at position 13; and 32% included Asp at position 14. The presence of Val (27%) or Glu (19%) at position 14 was among the most highly selected in the round 4 isolates, in comparison to their theoretical proportion (4% each) at position 14 in BAML.

The sequences in Table 14 are grouped according to their degree of difference from the core sequence (SEQ ID NO:436).

14-mer amino acid position														SEQ ID NO:
1	2	3	4	5	6	7	8	9	10	11	12	13	14	
A	n	w	y	D	s	L	T	k	L	w	l	p	d	consensus; 184
<b>A</b>	<b>N</b>	<b>W</b>	<b>Y</b>	<b>D</b>	<b>P</b>	<b>L</b>	<b>T</b>	<b>K</b>	<b>L</b>	<b>W</b>	<b>L</b>	<b>P</b>	<b>D</b>	186
A	N	W	Y	D	P	L	T	K	L	W	L	P	E	187
A	N	W	Y	D	P	L	T	K	L	W	L	P	G	188
A	N	W	Y	D	P	L	T	K	L	W	L	P	V	189
A	N	W	Y	D	P	L	T	K	L	W	L	S	D	190

A	N	W	Y	D	P	L	T	K	L	W	L	N	D	191
A	N	W	Y	D	P	L	T	K	L	W	L	P	T	192
A	N	W	Y	D	P	L	T	K	L	W	L	P	A	193
A	N	W	Y	D	P	L	T	K	L	W	L	P	N	194
A	N	W	Y	D	P	L	T	K	L	W	L	V	D	195
A	N	W	Y	D	P	L	T	K	L	W	L	H	D	196
A	N	W	Y	D	P	L	T	K	L	W	L	T	D	197
A	N	W	Y	D	P	L	T	K	L	W	L	P	H	198
A	N	W	Y	D	P	L	T	K	L	W	L	T	V	199
A	N	W	Y	D	P	L	T	K	L	W	L	L	D	200
A	N	W	Y	D	P	L	T	K	L	W	L	L	E	201
A	N	W	Y	D	P	L	T	K	L	W	L	H	E	202
A	N	W	Y	D	P	L	T	K	L	W	L	P	R	203
A	N	W	Y	D	P	L	T	K	L	W	L	A	D	204
A	N	W	Y	D	P	L	T	K	L	W	L	P	Y	205
A	N	W	Y	D	P	L	T	K	L	W	L	P	I	206
A	N	W	Y	D	P	L	T	K	L	W	L	I	D	207
A	N	W	Y	D	P	L	T	K	L	W	L	R	D	208
A	Y	W	Y	D	P	L	T	K	L	W	L	P	D	209
A	Y	W	Y	D	P	L	T	K	L	W	L	L	E	210
A	Y	W	Y	D	P	L	T	K	L	W	L	R	V	211
A	Y	W	Y	D	P	L	T	K	L	W	L	P	E	212
A	Y	W	Y	D	P	L	T	K	L	W	L	P	V	213
A	Y	W	Y	D	P	L	T	K	L	W	L	H	Q	214
A	Y	W	Y	D	P	L	T	K	L	W	L	P	A	215
A	Y	W	Y	D	P	L	T	K	L	W	L	R	V	216
A	Y	W	Y	D	P	L	T	K	L	W	L	P	G	217
A	Y	W	Y	D	P	L	T	K	L	W	L	R	Y	218
A	Y	W	Y	D	P	L	T	K	L	W	L	P	Y	219
A	Y	W	Y	D	P	L	T	K	L	W	L	L	Y	220
A	Y	W	Y	D	P	L	T	K	L	W	L	R	D	221
A	Y	W	Y	D	P	L	T	K	L	W	L	P	V	222
A	Y	W	Y	D	P	L	T	K	L	W	L	L	G	223
A	Y	W	Y	D	P	L	T	K	L	W	L	T	H	224
A	Y	W	Y	D	P	L	T	K	L	W	L	P	T	225
A	Y	W	Y	D	P	L	T	K	L	W	L	L	V	226
A	Y	W	Y	D	P	L	T	K	L	W	L	Y	Y	227
A	Y	W	Y	D	P	L	T	K	L	W	L	S	D	228
A	S	W	Y	D	P	L	T	K	L	W	L	P	A	229
A	S	W	Y	D	P	L	T	K	L	W	L	H	D	230
A	S	W	Y	D	P	L	T	K	L	W	L	P	G	231
A	S	W	Y	D	P	L	T	K	L	W	L	P	Q	232
A	S	W	Y	D	P	L	T	K	L	W	L	P	Y	233
A	S	W	Y	D	P	L	T	K	L	W	L	P	H	234
A	S	W	Y	D	P	L	T	K	L	W	L	P	V	235
A	S	W	Y	D	P	L	T	K	L	W	L	P	I	236
A	S	W	Y	D	P	L	T	K	L	W	L	P	E	237
A	F	W	Y	D	P	L	T	K	L	W	L	R	V	238
A	F	W	Y	D	P	L	T	K	L	W	L	P	E	239

A	F	W	Y	D	P	L	T	K	L	W	L	L	E	240
A	F	W	Y	D	P	L	T	K	L	W	L	P	V	241
A	I	W	Y	D	P	L	T	K	L	W	L	P	E	242
A	I	W	Y	D	P	L	T	K	L	W	L	P	D	243
A	I	W	Y	D	P	L	T	K	L	W	L	H	D	244
A	I	W	Y	D	P	L	T	K	L	W	L	T	D	245
A	I	W	Y	D	P	L	T	K	L	W	L	P	F	246
A	I	W	Y	D	P	L	T	K	L	W	L	L	D	247
A	I	W	Y	D	P	L	T	K	L	W	L	P	R	248
A	I	W	Y	D	P	L	T	K	L	W	L	P	A	249
A	I	W	Y	D	P	L	T	K	L	W	L	T	A	250
A	I	W	Y	D	P	L	T	K	L	W	L	A	V	251
A	I	W	Y	D	P	L	T	K	L	W	L	P	G	252
A	I	W	Y	D	P	L	T	K	L	W	L	R	V	253
A	I	W	Y	D	P	L	T	K	L	W	L	P	H	254
A	I	W	Y	D	P	L	T	K	L	W	L	R	E	255
A	I	W	Y	D	P	L	T	K	L	W	L	S	D	256
A	T	W	Y	D	P	L	T	K	L	W	L	P	A	257
A	T	W	Y	D	P	L	T	K	L	W	L	A	D	258
A	T	W	Y	D	P	L	T	K	L	W	L	T	S	259
A	T	W	Y	D	P	L	T	K	L	W	L	P	G	260
A	T	W	Y	D	P	L	T	K	L	W	L	P	Y	261
A	T	W	Y	D	P	L	T	K	L	W	L	S	G	262
A	T	W	Y	D	P	L	T	K	L	W	L	P	V	263
A	T	W	Y	D	P	L	T	K	L	W	L	P	D	264
A	D	W	Y	D	P	L	T	K	L	W	L	P	V	265
A	D	W	Y	D	P	L	T	K	L	W	L	P	K	266
A	D	W	Y	D	P	L	T	K	L	W	L	P	D	267
A	D	W	Y	D	P	L	T	K	L	W	L	P	E	268
A	D	W	Y	D	P	L	T	K	L	W	L	H	Q	269
A	E	W	Y	D	P	L	T	K	L	W	L	R	D	270
A	E	W	Y	D	P	L	T	K	L	W	L	P	D	271
A	E	W	Y	D	P	L	T	K	L	W	L	P	Y	272
A	L	W	Y	D	P	L	T	K	L	W	L	P	A	273
A	L	W	Y	D	P	L	T	K	L	W	L	P	D	274
A	L	W	Y	D	P	L	T	K	L	W	L	R	G	275
A	L	W	Y	D	P	L	T	K	L	W	L	L	G	276
A	M	W	Y	D	P	L	T	K	L	W	L	P	A	277
A	M	W	Y	D	P	L	T	K	L	W	L	Q	V	278
A	M	W	Y	D	P	L	T	K	L	W	L	L	G	279
A	A	W	Y	D	P	L	T	K	L	W	L	P	D	280
A	A	W	Y	D	P	L	T	K	L	W	L	A	D	281
A	A	W	Y	D	P	L	T	K	L	W	L	L	D	282
A	H	W	Y	D	P	L	T	K	L	W	L	T	D	283
A	H	W	Y	D	P	L	T	K	L	W	L	P	V	284
A	H	W	Y	D	P	L	T	K	L	W	L	H	D	285
A	H	W	Y	D	P	L	T	K	L	W	L	P	D	286
A	P	W	Y	D	P	L	T	K	L	W	L	H	D	287
A	P	W	Y	D	P	L	T	K	L	W	L	P	V	288









A	C	A	Y	D	A	L	T	K	L	C	L	P	A	409
A	N	W	Y	D	P	L	T	N	L	S	L	L	L	410
A	Y	W	Y	D	P	L	T	Q	L	S	L	L	V	411
A	Y	R	Y	D	A	L	T	G	L	W	L	L	Y	412
A	Y	W	N	D	P	L	T	K	L	K	L	R	L	413
A	Y	W	Y	D	P	L	T	Q	L	S	L	L	V	414
A	Y	R	Y	D	A	L	T	G	L	W	L	L	Y	415
A	Y	R	Y	D	S	L	T	N	L	W	L	L	Y	416
A	Y	W	Y	D	P	L	T	K	L	S	I	L	E	417
A	S	C	Y	D	P	L	T	K	L	C	F	P	V	418
A	F	W	F	D	P	L	T	G	L	W	L	L	E	419
1	2	3	4	5	6	7	8	9	10	11	12	13	14	
A	N	W	Y	D	P	L	T	K	L	W	L	P	D	186
A	H	W	Y	D	P	L	T	K	L	S	I	R	V	420
A	P	W	Y	D	S	L	T	K	L	W	F	P	S	421
A	N	C	Y	D	T	L	T	K	L	W	L	T	C	422
A	N	W	Y	D	S	L	T	K	L	S	L	P	D	423
A	Y	A	Y	D	F	L	T	Q	L	S	L	P	D	424
A	F	R	Y	D	S	L	T	G	L	W	L	R	Y	425
A	N	C	Y	D	S	L	T	K	L	W	L	P	C	426
A	N	G	Y	D	L	L	T	N	L	S	V	S	D	427
A	N	W	Y	D	P	L	T	R	L	W	I	P	V	428
A	L	K	F	D	Y	L	T	K	L	W	L	P	D	429
A	Y	R	Y	D	S	L	T	K	L	W	L	P	G	430
A	Y	C	Y	D	S	L	T	K	L	W	I	P	D	431
A	S	W	E	D	S	L	T	K	L	W	L	S	K	432
A	Y	W	Y	D	S	L	T	G	L	S	L	L	V	433
A	Y	W	Y	D	P	L	T	Y	L	R	L	R	V	434
A	K	C	Y	D	S	L	T	N	L	W	L	C	D	435

Nearly all of the ELISA signals of the BAML isolates were higher than those isolated in the initial screen (see Example 1). For comparison, peptide 453-01-B07 (SEQ ID NO:31) ( $K_D = 700\text{nM}$ ) was used as a reference (positive control). Negative control MAEX (M13 phage with no insert) did not bind b-BLyS at any concentration tested.

For direct phage ELISA, the signal measured is a reflection of the ability of a set number of phage to bind to various concentrations of b-BLyS. Peptides tested by the direct phage ELISA assay were chosen based on high affinity for BLyS as determined in the indirect phage ELISA assay. For this assay, Immulon-2HB plates were coated with 0 or 1000ng anti-Fd antibody (Sigma, St. Louis, MO). After washing (PBS-Tween-20), phage dilutions were added to saturate the available antibody and incubated for 1 hour, washed, then incubated with 100  $\mu\text{l}$  of 10-fold dilutions of b-BLyS (0-1 $\mu\text{g/ml}$ ) for 1 hour at room temperature. Streptavidin-HRP

(1:1000 in PBS-tween-20; Endogen, Woburn, MA) was added to the wells and incubated for 1 hour, developed using TMB and reading at OD 630 nm.

#### Determination of BAML Peptide $K_D$ by Fluorescence Anisotropy.

Several peptides containing the 10-mer core structural motif or single-position variants of that motif identified by sequence analysis were synthesized with a short Gly-Gly-Lys linker sequence and the C-terminal lysine was labeled with fluorescein. These peptides, shown in Table 15, below, were synthesized by solid phase synthesis for determination of dissociation constant with respect to BLyS. The DX815 and DX876 polypeptides were derived from DX814 (SEQ ID NO:186) by deletion of two N-terminal amino acids or the two amino acids N-terminal and C-terminal to the core peptide at (positions 3-12). DX816, DX817, DX819, and DX822 correspond to other BAML isolates (SEQ ID NOs:189, 309, 353, 327, respectively). DX818 corresponds to isolate SEQ ID NO:340, except that Asn has been substituted for Met at position 2. The  $K_D$  of several BLyS binding BAML peptides was determined by fluorescence anisotropy, performed as previously described. The sequence of DX822 without the -GGK linker (see SEQ ID NO:327) matches the BAML template sequence (see Table 11). The BAML consensus sequence found in DX822 resulted in a more than 10-fold improvement in binding affinity for BLyS, as compared to one of the highest affinity binders isolated in the initial screen (453-01-B07, SEQ ID NO:31).

Table 15: Dissociation Constants of Synthetic BLyS-binding Polypeptides

Peptide	Sequence	SEQ ID NO:	$K_D$ (nM)
DX814	Ac-ANWYDPLTKLWLPDGGK-fitc	437	$26 \pm 7$
DX815	Ac-WYDPLTKLWLPDGGK-fitc	438	$31 \pm 13$
DX876	Ac-WYDPLTKLWLGGK-fitc	439	$171 \pm 90$
DX816	Ac-ANWYDPLTKLWLPVGGK-fitc	440	$44 \pm 15$
DX817	Ac-ANWFDPLTKLWLPDGGK-fitc	441	$32 \pm 26$
DX818	Ac-ANWYDPLTKLSLPDGGK-fitc	442	$342 \pm 108$
DX819	Ac-ANWYDPLTKLWFPDGGK-fitc	443	$69 \pm 38$
DX822	Ac-ANWYDSLTKLWLPDGGK-fitc	444	$79 \pm 54$

Analysis of the BAML isolates revealed a lack of sequence conservation at position 2 (varied in the BAML template, see Table 11). To examine whether the N-terminal residues at positions 1 and 2 in the BAML sequence were necessary for binding to BLYS, a truncated version of DX814 comprising only residues 3-14 (DX815; see Table 15) was synthesized and analyzed by fluorescence anisotropy. The  $K_D$  for DX815 was indistinguishable from that of DX814, suggesting that residues 1-2 are not required for high affinity binding to BLYS. Further truncation of DX814 to the minimal core (residues 1-10, DX876) increased the  $K_D$  to 171 nM, indicating a contribution from Pro at position 13 and/or Asp at position 14 of the 14-mer to high affinity BLYS binding. Substitution of Val in DX816 at that position had little effect on the  $K_D$  (see Table 15). In comparing the BLYS-binding polypeptide DX221 (Ac-WTDSLTLGLWFPDGGPGPEGGGK;  $K_D$  = 3  $\mu$ M; SEQ ID NO:168) with the BAML peptide closest in sequence (DX819, Ac-ANWYDPLTKLWFPDGGK;  $K_D$  = 69 nM; SEQ ID NO:443), differences are seen at three positions 4 (T  $\rightarrow$  Y), 6 (S  $\rightarrow$  P), and 9 (G  $\rightarrow$  K), indicating the contribution of these residues in binding affinity.

The synthesized BAML peptides exhibited  $K_D$  values in the low nanomolar range, two orders of magnitude lower than primary isolate-derived peptides (see Example 1). Phenylalanine substitutions (F<sub>4</sub> $\rightarrow$ Y<sub>4</sub>; F<sub>12</sub> $\rightarrow$ L<sub>12</sub>; Table 17) were the most common minor variations to the core sequence and these changes failed to significantly affect the dissociation constants of the synthesized peptides. A change at position 11 (W<sub>11</sub> $\rightarrow$ S<sub>11</sub>; DX818), however, resulted in an approximately 10-fold decrease in affinity compared to DX814.

Following the foregoing description, the characteristics important for affinity binding polypeptides permitting detection or separation of BLYS or BLYS-like polypeptides (BLYS target protein) in or from any solution can be appreciated. Additional binding polypeptide embodiments of the invention and alternative methods adapted to a particular solution or feed stream will be evident from studying the foregoing description. For instance, any spacer or linker sequences associated with BLYS binding polypeptides discussed above may be removed or substituted to yield additional BLYS binding polypeptides of this invention. Also, very high affinity polypeptide BLYS target binders suitable for in vivo therapeutic applications may be prepared, e.g., by selecting among the peptides isolated from the BAML, by selecting similar polypeptides under similarly stringent conditions from BAML or other peptide library, or by designing a polypeptide binding molecule following the descriptions above, e.g., of important

structural motifs contributing to BLYS binding properties. All such embodiments and obvious alternatives are intended to be within the scope of this invention, as defined by the claims that follow.

The publications referred to above are hereby incorporated by reference in their  
5   entireties.